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ENVIRONMENTAL ASSESSMENT AND OVERVIEW OF BIOTECHNOLOGY PROCESS APPLICATIONS

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Bioengineering	Genetics	Stationary Sources	06B	
Assessments		Biotechnology	14B	
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ABSTRACT

This report presents an overview of biotechnology processes, the waste streams associated with those processes, and the effectiveness of current control technologies in treating process waste streams. Biotechnology may be defined more broadly than in this report; hence the term biotechnology is defined as processes which employ microbial cultures to produce a product or to effect a change in the environment. Commercial applications are divided into contained product manufacturing processes (fermentation) and semi-contained and uncontained processes. "Fermentation" is herein used as industrial jargon; i.e., any large scale process catalyzed by microbes/enzymes. Scientifically, fermentation may be defined differently, for example, as distinguished from respiration.

Waste streams associated with biotechnology processes are identified and characterized in terms of both chemical and biological contaminants. The effectiveness of current control technologies both in treatment of the microbe and in treatment of chemical contaminants is also addressed. Additionally, the potential impacts on the effectiveness of conventional waste treatment technologies due to the presence of the microbe in process waste streams are discussed.

In general, few data were found as to the specific characterization of waste streams or on the effectiveness of current control technologies. A more complete characterization of these wastes is needed to adequately address their potential impacts to the environment. The full extent of potential impacts associated with release of viable microbes is generally unknown. A realistic hazard assessment of impacts associated with microbial release is needed in order to define the appropriate level of control technology which should be applied.

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SECTION 1

SUMMARY AND CONCLUSIONS

1.1 OVERVIEW

The term biotechnology as used in this report refers to commercial and industrial processes which employ microbial cultures/enzymes or higher order organisms to produce a product or to effect a specific physical or chemical change. This report provides an overview of biotechnology processes, identifies the potential waste streams associated with these processes and their potential impacts on the environment, and addresses the effectiveness of current control technologies for treatment of process waste streams. Agricultural applications of biotechnology and the use of higher order organisms are not considered in detail in this report nor is biological treatment of municipal sewage or typical organic/inorganic-laden wastewaters.

Figure 1 presents an overall schematic of the biotechnology industry. The first phase of all biotechnology processes is the development of a microbial culture or organism that will produce the desired effect in subsequent process applications. This may be accomplished through natural selection, induced mutations, recombinant DNA, and cell fusion methods. Once the appropriate microorganism is identified, the culture is isolated through selection and purification techniques and, subsequently, encouraged to reproduce to yield industrial quantities of microbial cultures for commercial applications.

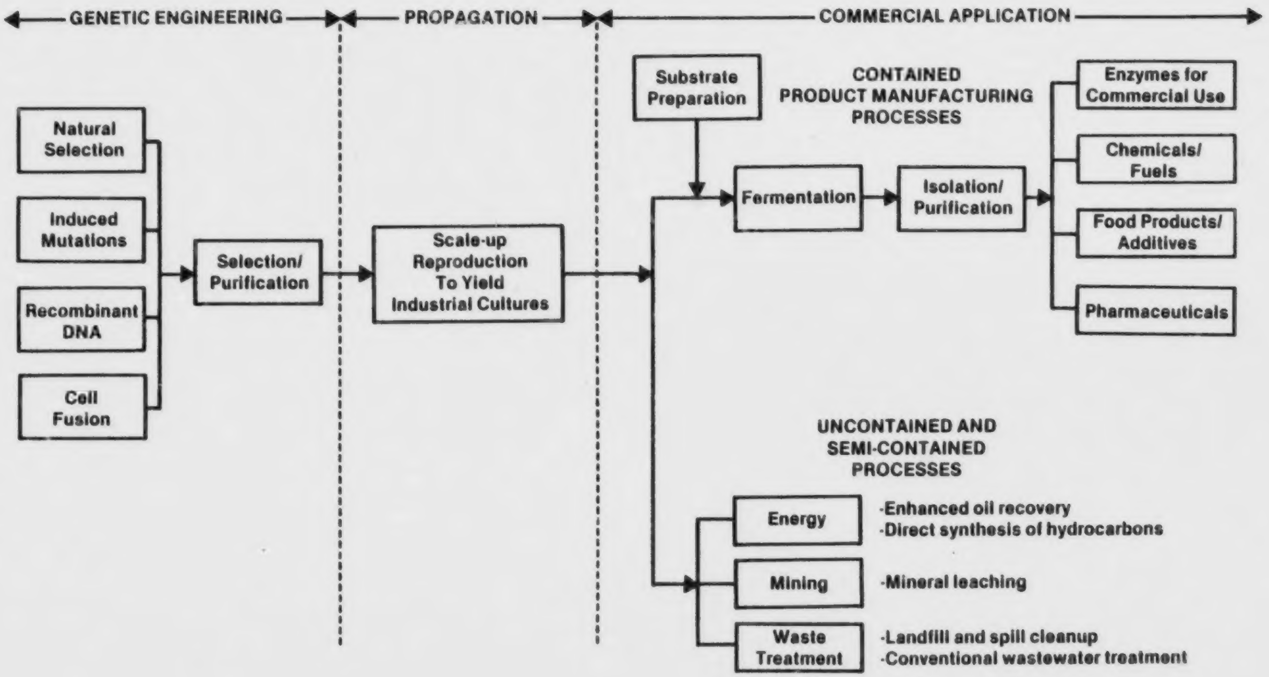

GENETIC ENGINEERING
PROPAGATION
COMMERCIAL APPLICATION
Natural Selection
Induced Mutations
Recombinant DNA
Cell Fusion
Selection/ Purification
Scale-up Reproduction To Yield Industrial Cultures
Substrate Preparation
CONTAINED PRODUCT MANUFACTURING PROCESSES
Fermentation
Isolation/ Purification
Enzymes for Commercial Use
Chemicals/ Fuels
Food Products/ Additives
Pharmaceuticals
UNCONTAINED AND SEMI-CONTAINED PROCESSES
Energy
Mining
Waste Treatment
-Enhanced oil recovery
-Direct synthesis of hydrocarbons
-Mineral leaching
-Landfill and spill cleanup
-Conventional wastewater treatment

Figure 1. Characterization of the Biotechnology Industry.

1.2 COMMERCIAL APPLICATIONS

Potential commercial applications of biotechnology can be divided into two general categories loosely defined as: a) contained product manufacturing processes, and b) uncontained and semi-contained processes.

Contained product manufacturing processes are analogous to in-plant chemical processing and use the microbe or its enzymes to convert a feedstock (substrate) to a useful product by fermentation. Fermentation processes may be aerobic or anaerobic; and the desired product may be the converted substrate or increased quantities of the actual microbial cells. Products currently produced by fermentation processes include organic acids, alcohols, methane, amino acids, vitamins, various medicinals, enzymes, polymers, surfactants, hydrocarbons, and single cell protein.

There are three major process components in contained product manufacturing processes: substrate preparation, fermentation, and product isolation and purification. Generalized flow diagrams for each of these process components are presented in Figures 2 through 4, respectively.

Substrate preparation may involve pretreatment of the feedstock material to convert it to a form suitable for microbial conversion, sterilization, nutrient addition, and pH adjustment.

During fermentation the prepared substrate is contacted with the microbe and converted to structurally related compounds by one or more enzymes supplied by the microbial cells. There are two types of fermentation processes: submerged and immobilized. In the former, the culture or enzyme is completely mixed and maintained in suspension with the substrate. In immobilized processes the culture or enzyme is supported or encapsulated in a nutritionally inert matrix and the substrate is passed over the fixed structure.

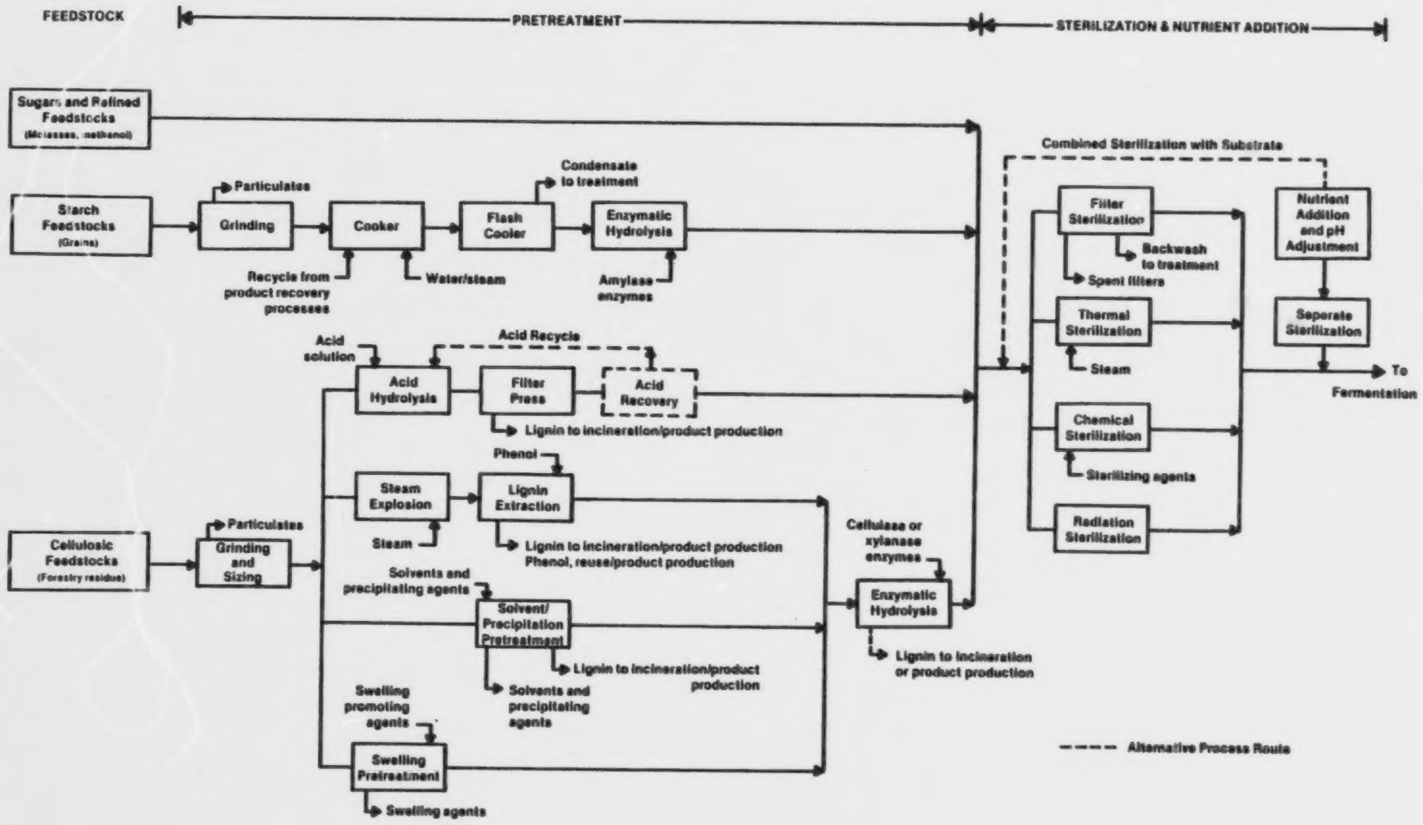

FEEDSTOCK
PRETREATMENT
STERILIZATION & NUTRIENT ADDITION
Sugars and Refined Feedstocks (Molasses, methanol)
Starch Feedstocks (Grains)
Particulates
Grinding
Cooker
Recycle from product recovery processes
Water/steam
Flash Cooler
Condensate to treatment
Enzymatic Hydrolysis
Amylase enzymes
Acid Recycle
Acid solution
Acid Hydrolysis
Filter Press
Acid Recovery
Lignin to incineration/product production
Phenol
Steam Explosion
Steam
Lignin Extraction
Lignin to incineration/product production
Phenol, reuse/product production
Cellulosic Feedstocks (Forestry residue)
Particulates
Grinding and Sizing
Solvents and precipitating agents
Solvent/ Precipitation Pretreatment
Lignin to incineration/product production
Solvents and precipitating agents
Swelling promoting agents
Swelling Pretreatment
Swelling agents
Cellulase or xylanase enzymes
Enzymatic Hydrolysis
Lignin to incineration or product production
Combined Sterilization with Substrate
Filter Sterilization
Backwash to treatment
Spent filters
Thermal Sterilization
Steam
Chemical Sterilization
Sterilizing agents
Radiation Sterilization
Nutrient Addition and pH Adjustment
Separate Sterilization
To Fermentation
Alternative Process Route

Figure 2. Substrate Preparation Processes.

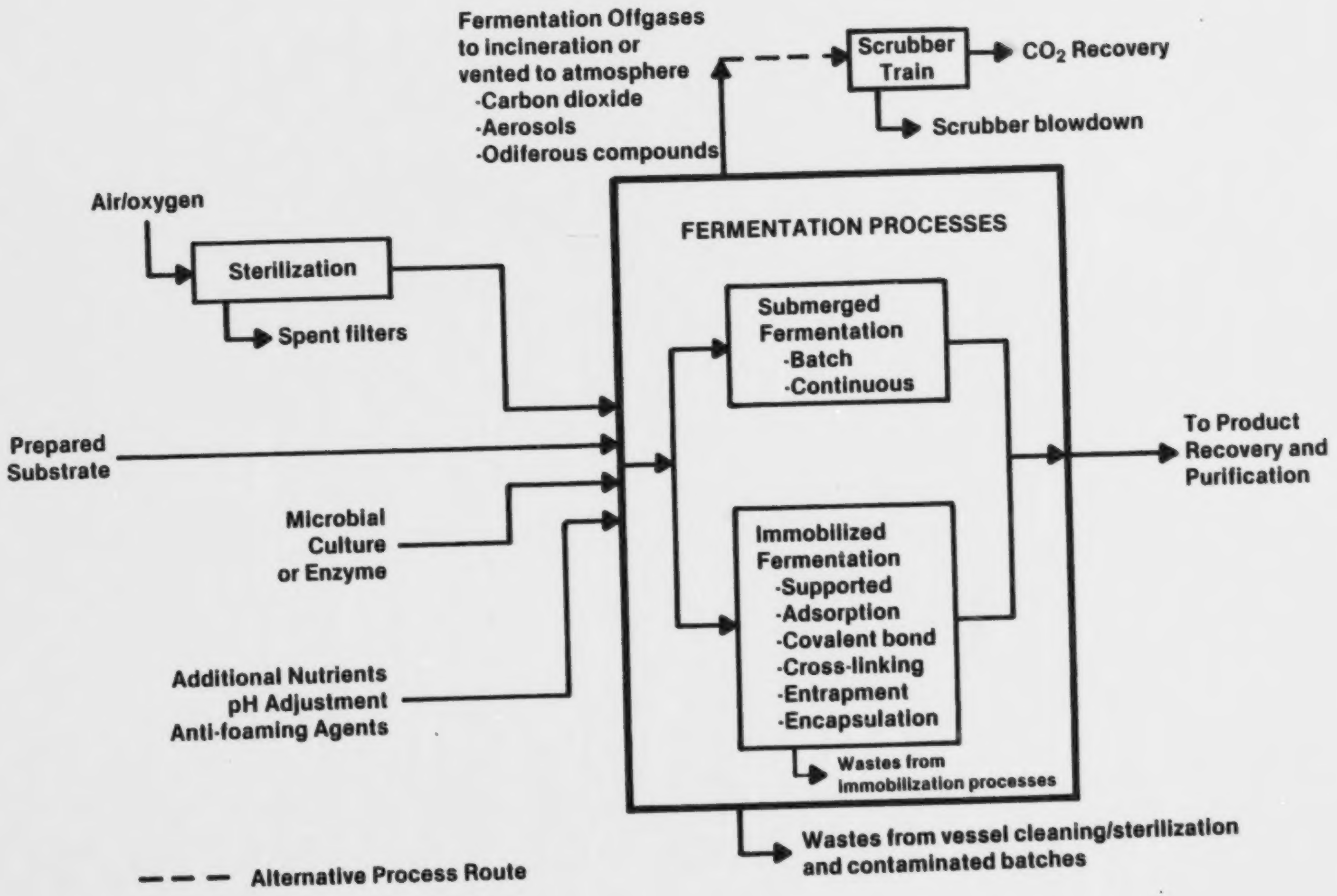

Fermentation Offgases
to incineration or
vented to atmosphere
-Carbon dioxide
-Aerosols
-Odiferous compounds
Scrubber Train
CO$_2$ Recovery
Scrubber blowdown
Air/oxygen
Sterilization
Spent filters
Prepared Substrate
Microbial Culture or Enzyme
Additional Nutrients
pH Adjustment
Anti-foaming Agents
FERMENTATION PROCESSES
Submerged Fermentation
-Batch
-Continuous
Immobilized Fermentation
-Supported
-Adsorption
-Covalent bond
-Cross-linking
-Entrapment
-Encapsulation
Wastes from immobilization processes
To Product Recovery and Purification
Wastes from vessel cleaning/sterilization and contaminated batches
Alternative Process Route

Figure 3. Fermentation Processes.

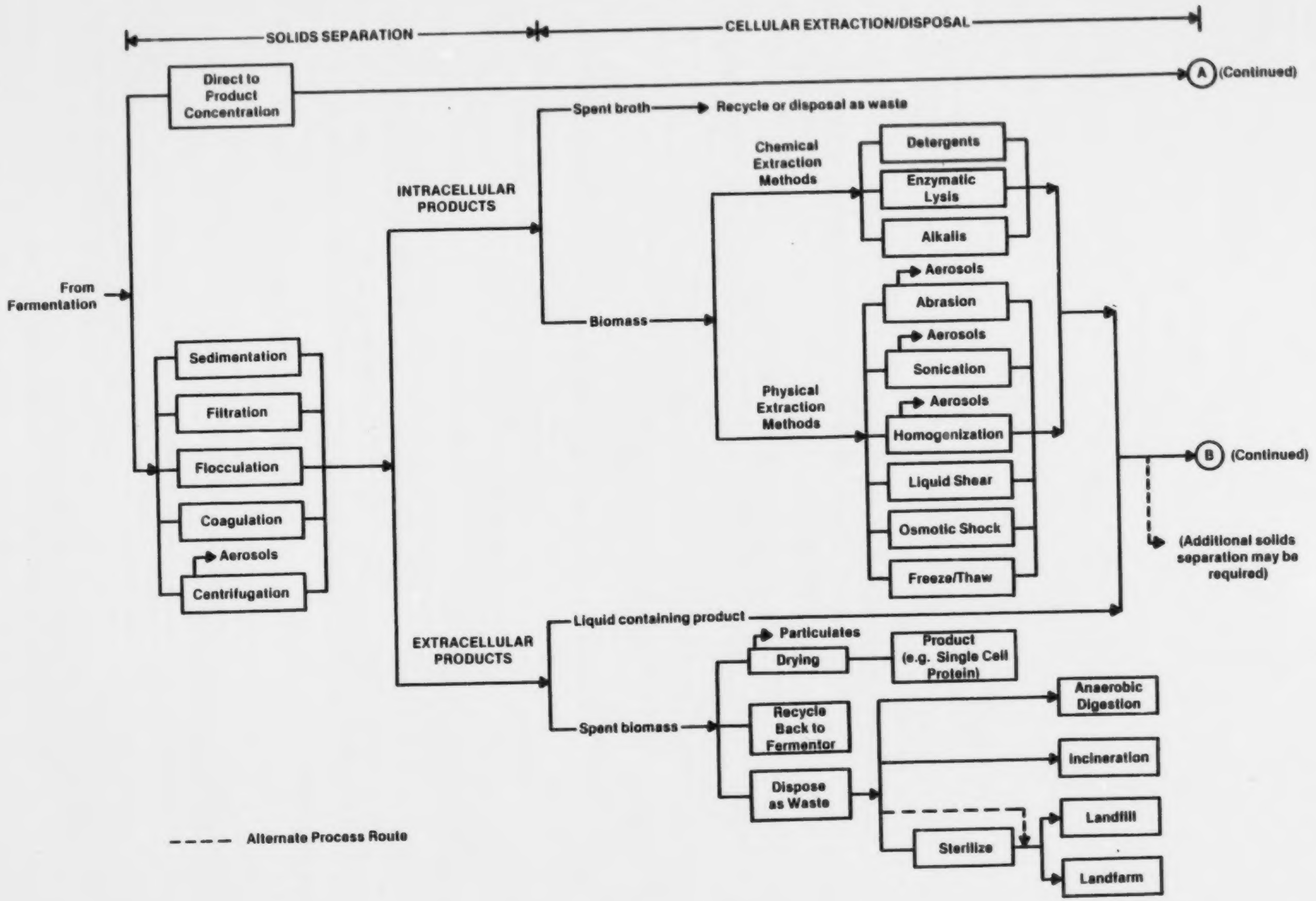

SOLIDS SEPARATION
CELLULAR EXTRACTION/DISPOSAL
From Fermentation
Direct to Product Concentration
A (Continued)
Sedimentation
Filtration
Flocculation
Coagulation
Aerosols
Centrifugation
INTRACELLULAR PRODUCTS
Spent broth
Recycle or disposal as waste
Biomass
Chemical Extraction Methods
Detergents
Enzymatic Lysis
Alkalis
Physical Extraction Methods
Aerosols
Abrasion
Aerosols
Sonication
Aerosols
Homogenization
Liquid Shear
Osmotic Shock
Freeze/Thaw
B (Continued)
(Additional solids separation may be required)
EXTRACELLULAR PRODUCTS
Liquid containing product
Particulates
Drying
Product (e.g. Single Cell Protein)
Spent biomass
Recycle Back to Fermentor
Dispose as Waste
Anaerobic Digestion
Incineration
Sterilize
Landfill
Landfarm
Alternate Process Route

Figure 4. Product Recovery and Purification Processes. (Continued next page.)

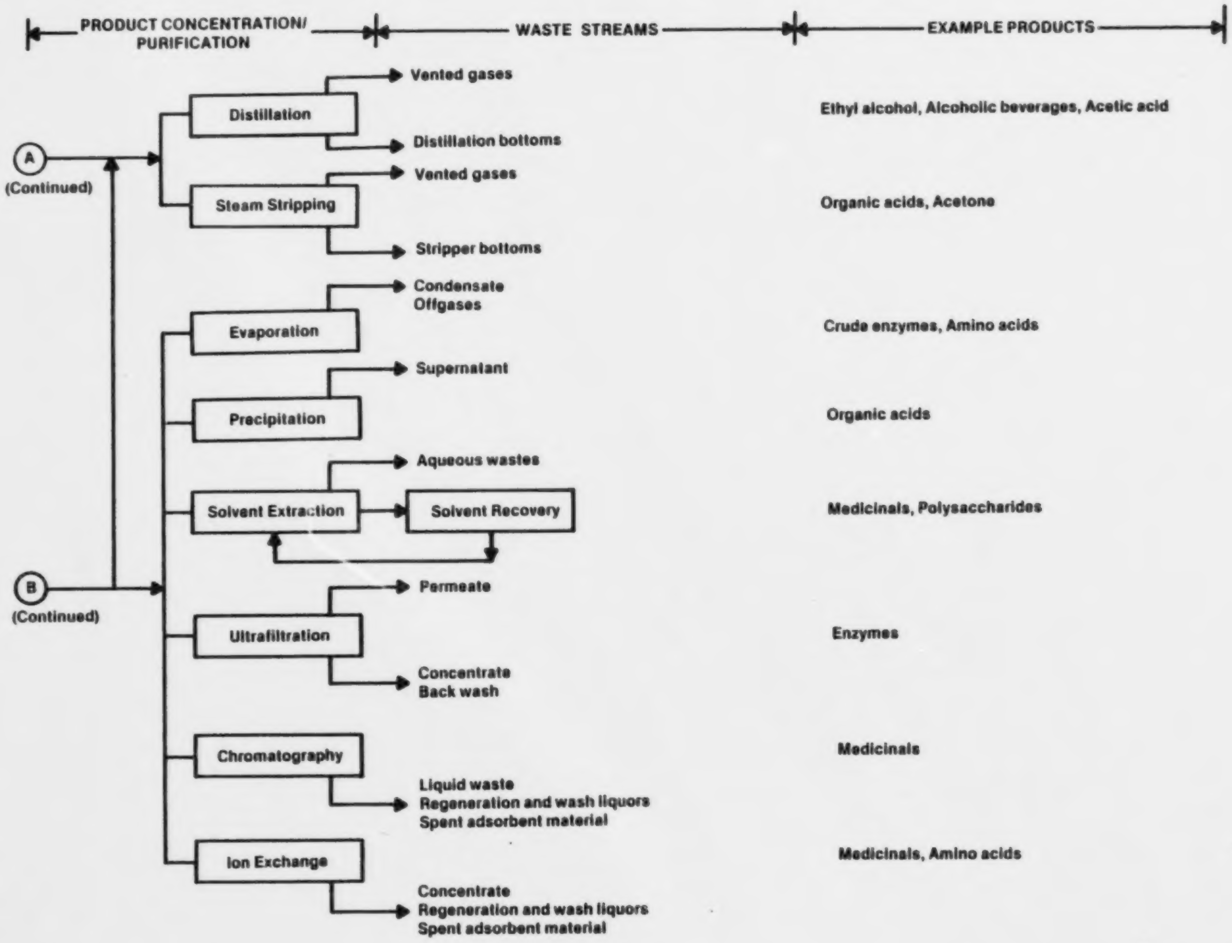

PRODUCT CONCENTRATION/ PURIFICATION
WASTE STREAMS
EXAMPLE PRODUCTS
A
(Continued)
B
(Continued)
Distillation
Vented gases
Distillation bottoms
Ethyl alcohol, Alcoholic beverages, Acetic acid
Steam Stripping
Vented gases
Stripper bottoms
Organic acids, Acetone
Evaporation
Condensate
Offgases
Crude enzymes, Amino acids
Precipitation
Supernatant
Organic acids
Solvent Extraction
Solvent Recovery
Aqueous wastes
Medicinals, Polysaccharides
Ultrafiltration
Permeate
Concentrate
Back wash
Enzymes
Chromatography
Liquid waste
Regeneration and wash liquors
Spent adsorbent material
Medicinals
Ion Exchange
Concentrate
Regeneration and wash liquors
Spent adsorbent material
Medicinals, Amino acids

Figure 4. (Continued)

The final process involves recovery of the product through a variety of separation technologies. These processes typically consist of solids separation to isolate the broth from the solid culture, cell disruption processes if the product is formed within the cell as opposed to excreted through the cell wall into the broth, and a variety of product concentration/purification processes such as distillation, steam stripping, and precipitation.

Whereas contained product manufacturing processes involve use of the microbe in a closed, somewhat controlled environment, uncontained and semi-contained processes may involve direct release of the microbe to the environment. These processes use the microbe to recover a product by non-fermentation processes such as adsorption or to effect a specific physical or chemical change in the environment. Examples of such applications include use of the organism to extract minerals from ore or from metal bearing wastes, to enhance oil recovery, or to clean up chemical spills and uncontrolled hazardous waste sites.

With the exception of metals extraction from ore, the majority of these processes have not currently been applied on a commercial scale but, rather, are in various stages of laboratory or field testing. The waste streams generated from these processes have not been well defined and present special environmental concerns since they involve direct release of the microbe to the environment with unpredictable results.

1.3 ENVIRONMENTAL CONCERNS

The major environmental concern associated with biotechnology processes is the potential for harmful effects from the release of viable microorganisms both in the workplace and in the environment. Other potential environmental impacts center around the discharge of high strength organic effluents, particulate and organic atmospheric emissions, and disposal of sludges from biological waste treatment and product recovery and purification processes.

In contained process applications, viable microorganisms will most probably be present in all of the fermentation and product recovery and purification waste streams with spent biomass representing the largest discharge of viable microorganisms. The possible hazards associated with release of these microbes include:

- adverse effects of the microbe or its products on the natural balance,
- allergic reactions in plant workers and in the general population,
- the potential for some organisms to cause disease in higher life forms including humans, and
- the potential for the organism to change during the process or after release, thus invalidating previous testing which demonstrated the process' safety.

The full extent of potential hazards associated with release of any viable organism, and in particular genetically engineered microorganisms, generally is unknown.

1.4 WASTE STREAMS AND CONTROL TECHNOLOGIES

The waste steams associated with contained product manufacturing processes as well as the current methods of treatment or disposal are presented in Tables 1 through 3. Because of the highly proprietary nature of most fermentation processes and the lack of commercial-scale applications of uncontained processes, little or no data are available as to the specific characteristics of these waste streams or the effectiveness of current control technologies. Classes of contaminants which may be expected in these wastes include refractory organics, organic acids, alcohols, hydrocarbons, metals, and pesticides.

Most control technologies currently employed in biotechnology processes are aimed at treatment of chemical contaminants in waste streams rather than control of biological contaminants. With the exception of incineration, these

TABLE 1. WASTE STREAMS FROM SUBSTRATE PREPARATION

Process Unit	Waste Stream	Potential Contaminants	Current Treatment/ Disposal Methods
PRETREATMENT			
Sugars and Refined Substrates	no waste stream identified	-	-
Starch Substrates			
Grinding	particulate emissions	dust	cyclones, baghouses
Flash Cooler	condensate/water	BOD $\sim$ 10 mg/L TS $\sim$ 80 mg/L TSS $\sim$ 10 mg/L	conventional biological wastewater treatment
Cellulosic Substrates			
Grinding	particulate emissions	dust	cyclones, baghouses
Acid Hydrolysis	filter cake/lignin, ash	polymers aromatics	incineration used in production of products
Enzyme Hydrolysis			
steam explosion	No data	No data	No data
lignin extraction	lignin residue	spent solvent phenols polymers aromatics	used in production of products incineration
solvent extraction/precipitation	spent solvent and precipitating agents	phosphoric acid cadoxen hydrazine sulfuric acid ferric tartrate cuprammonium sulfate	No data
	lignin	polymers aromatics	used in production of products incineration
swelling pretreatment	swelling agents	sodium hydroxide organic bases (amines) salts	No data
	lignin	polymers aromatics	used in production of products incineration
STERILIZATION			
Filtration	spent filters	viable microorganisms feedstock residual	incineration landfill
	backwash	viable microorganisms high solids feedstock residual	conventional biological wastewater treatment
Thermal, Chemical, Radiation	No data	No data	No data

TABLE 2. WASTE STREAMS FROM FERMENTATION PROCESSES

Fermentation Process	Waste Streams	Potential Contaminants	Current Treatment/ Disposal Methods
AIR/OXYGEN STERILIZATION	spent filters	particulates and microbes found in ambient air	no data
FERMENTATION	offgases	carbon dioxide methane aerosols volatilized product microbe/enzymes odiferous compounds	vented to atmosphere scrubbers CO_2 recovery methane recovery incineration
	offgas filtration	spent filters viable microorganisms	no data
	scrubber blowdown	absorption chemicals light soluble organics organic oils and waxes	conventional biological wastewater treatment
	vessel cleaning wastes	detergents biocides viable microbes/enzymes feedstock residue product residue intermediate product residue nutrients	conventional biological wastewater treatment
	contaminated batches	high BOD, COD, TSS viable microbes/enzymes microbial virus feedstock product intermediate products nutrients	conventional biological wastewater treatment
IMMOBILIZATION PROCESSES			
adsorption	solution wash	no data (enzymes)	no data
covalent bonding cross-linking entrapment encapsulation	spent microbe/support matrix	microbes/enzymes polymers gels other supports	no data

TABLE 3. WASTE STREAMS ASSOCIATED WITH PRODUCT RECOVERY AND PURIFICATION

Unit	Waste Stream	Potential Contaminants	Current Treatment/ Disposal Methods
SOLIDS SEPARATION			
General	spent broth	unused organic substrate substrate contaminants (e.g., pesticides) unused nutrients residual product product intermediates viable microorganisms color	conventional biological wastewater treatment with pretreatment for specific pollutant removal
	spent biomass	concentrated viable microorganisms spent broth contaminants	recovered as by-product sterilization anaerobic digestion landfarming landfill incineration
Centrifugation	aerosols	viable microorganisms/ enzymes volatile organics	containment/treatment of air emissions by filtration/sterilization
CELLULAR EXTRACTION			
Abrasion, Sonication, Homogenization	aerosols	viable microorganisms/ enzymes volatile organics	containment/treatment of air emissions by filtration/sterilization
PRODUCT CONCENTRATION/ PURIFICATION			
Distillation	distillation bottoms	unused organic substrate substrate contaminants (e.g., pesticides) unused nutrients residual product product intermediates viable microorganisms/ enzymes cellular debris (proteins, lipids) color high BOD, COD, TSS	conventional biological wastewater treatment with pretreatment for specific pollutant removal
	vented gases	volatile organics	vented to atmosphere incinerated flared utilized as air feed to boilers
Steam Stripping	stripper bottoms	Same as distillation bottoms	conventional biological wastewater treatment with pretreatment for specific pollutant removal
	vented gases	volatile organics	vented to atmosphere incinerated flared utilized with air feed to boilers

(Continued)

TABLE 3. (Continued)

Unit	Waste Stream	Potential Contaminants	Current Treatment/ Disposal Methods
Evaporation	condensate	volatile organics and inorganics	steam stripping followed by conventional biological wastewater treatment
	offgases	volatile organics	incinerated flared utilized with air feed to boilers
Precipitation	supernatant	Same as distillation bottoms plus residual precipitating agents (Fe, Al, Cu, Zn, Ca) Reagents used for cellular extraction (detergents, enzymes, alkalies)	conventional biological wastewater treatment may require pretreatment to adjust pH or precipitate residual metals
Solvent Extraction	aqueous phase waste stream	Same as distillation bottoms plus residual solvent and reagents used in cellular extraction (detergents, enzymes, alkalies)	steam stripping to remove residual solvent conventional biological wastewater treatment
Ultrafiltration	permeate	Same as distillation bottoms, low solids	conventional biological wastewater treatment with pretreatment for specific pollutant removal
	concentrate	Same as distillation bottoms, potentially high solids	conventional biological wastewater treatment with pretreatment for specific pollutant removal
	backwash	Same as distillation bottoms, high solids	conventional biological wastewater treatment with pretreatement for specific pollutant removal
	spent filter	filter media, high solids, viable microorganisms	incineration
Chromatography	liquid waste	Same as distillation bottoms	conventional biological wastewater treatment with pretreatment for specific pollutant removal
	regeneration and wash liquors	solvents, acids, bases, residual product	no data
	spent adsorbent material	adsorbent product residue	no data

(Continued)

TABLE 3. (Continued)

Unit	Waste Stream	Potential Contaminants	Current Treatment/ Disposal Methods
Ion Exchange	concentrate	Same as distillation bottoms	conventional biological wastewater treatment with pretreatment for specific pollutant removal
	regeneration and wash liquors	acids, bases, residual product	no data
	spent adsorbent material	resins	no data

technologies have little or no impact on the microbe contained in process discharges. Additionally, these controls may not be adequate for the control of some chemical contaminants.

Process effluents are primarily treated by conventional biological treatment with pretreatment of isolated waste streams for specific pollutant removal as required. While this treatment should be adequate for control of BOD, COD, and TSS, refractory organics or metals may pass through the system or accumulate in the waste sludge. Additionally, biological wastewater treatment will have no effect on the process microbe. The microbe will be part of the mixed liquor and discharged to the environment with the waste sludge and in the treated effluent. The presence of the microbe in process wastewaters may also adversely affect the performance of other treatment technologies such as metals precipitation.

Control technologies for air emissions include conventional particulate controls, such as cyclones, wet scrubbers, and baghouses; incineration; flaring; and CO_2 or methane recovery. However, process offgases are often vented directly to the environment and may contain hydrocarbons harmful to human health. Incineration may provide for destruction of the viable process microbes if the system is designed to achieve sterilization. In general, however, the control technologies, when applied, do not afford any treatment of biological contaminants. Additionally, aerosol releases provide an excellent method for transport of the microbe to the general environment.

Disposal of solid waste is accomplished primarily through landfilling or landfarming and often represents direct release of the microbe to the environment. Sludges from biological waste treatment may be anaerobically digested with the residuals sold as animal feed. However, this waste may contain refractory organics or metals which have accumulated in the sludge. Spent solvents or sludges from some product recovery and purification processes (e.g., metals precipitation) may require disposal under the Resource Conservation and Recovery Act (RCRA).

Few data were found on current practices specifically designed for control of the process microbe. Spent biomass represents the largest volume and highest concentration of microbial waste. In some processes (e.g., ethanol production), this stream is dried and sold as animal feed. However, due to potential hazards associated with the microbe or residual chemical contaminants, this practice may not be appropriate for all or even most fermentation processes.

Current methods aimed at control of the microbe include:

- reduction of microbe viability by chemical treatment, heat sterilization, or incineration;
- containment ranging from basic enclosure and venting with treatment of the vented gases to the use of filters capable of retaining microorganisms and complete isolation;
- use of immobilized fermentation processes which retain the culture in the fermenter;
- genetically engineering an enfeebling mechanism in the microbe which would prevent its survival in the open environment; and
- nutrient denial.

While control technologies to contain or treat the process microbe are available, the extent of their effectiveness and their applicability to large-scale processes is unknown.

1.5 GENERAL CONCLUSIONS

As previously stated, very few data were available on the specific characteristics of biotechnology process waste streams or on the effectiveness of current treatment technologies. The extent of use and the need for more stringent controls, both for biological and chemical contaminants, will be dependent on adequate characterization of process wastes. A special need exists for a realistic hazard assessment procedure for microbial releases. Such a procedure would define the appropriate levels of controls necessary both for the workplace and the general environment.

SECTION 2

INTRODUCTION AND OVERVIEW

The term biotechnology as used in this report refers to commercial and industrial processes which employ microbial cultures/enzymes or higher order organisms to produce a product or to effect a specific physical or chemical change. This report provides an overview of biotechnology processes and identifies the potential waste streams associated with those processes. A discussion on waste stream characteristics that may have adverse effects on public health and the environment and the effectiveness of current control technologies is also presented.

Figure 5 presents an overall schematic of the biotechnology industry. By definition, the first phase of all biotechnology processes is the development of a microbial culture or organism that will produce the desired effect in subsequent process applications. The microbe may be selected from those that occur in nature or may be "produced" through genetic manipulations such as induced mutation, recombinant DNA, or cell fusion techniques. Once the appropriate microorganism is identified, a pure culture is developed through selection and purification processes. This culture can then be encouraged to reproduce to yield industrial quantities of microbial cultures for commercial applications.

Potential commercial applications can be divided into two general categories loosely defined as: a) contained product manufacturing processes and b) uncontained and semi-contained process applications. Contained product manufacturing processes are analogous to in-plant chemical processing and involve use of the microbe or its enzymes to convert a feedstock (substrate)

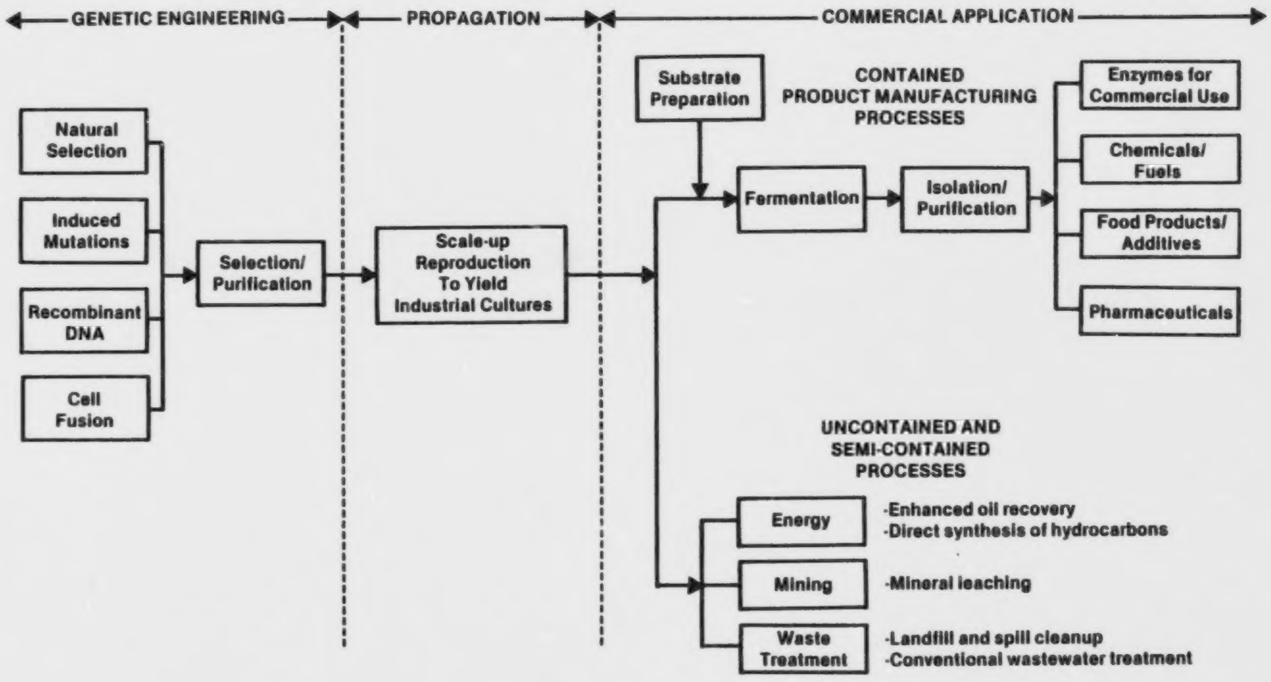

GENETIC ENGINEERING
PROPAGATION
COMMERCIAL APPLICATION
Natural Selection
Induced Mutations
Recombinant DNA
Cell Fusion
Selection/ Purification
Scale-up Reproduction To Yield Industrial Cultures
Substrate Preparation
CONTAINED PRODUCT MANUFACTURING PROCESSES
Fermentation
Isolation/ Purification
Enzymes for Commercial Use
Chemicals/ Fuels
Food Products/ Additives
Pharmaceuticals
UNCONTAINED AND SEMI-CONTAINED PROCESSES
Energy
-Enhanced oil recovery
-Direct synthesis of hydrocarbons
Mining
-Mineral leaching
Waste Treatment
-Landfill and spill cleanup
-Conventional wastewater treatment

Figure 5. Characterization of the Biotechnology Industry.

to a useful product by fermentation processes. By this definition, all contained product manufacturing processes are considered fermentation processes with fermentation defined as the aerobic or anaerobic breakdown of an organic substrate to form an organic product by microbial action. It should be noted that this definition of biotechnology is not limited to conversion of unrefined organic substrates such as grains and forestry residue; substrates may and often do consist of refined organic chemicals such as ethanol or various organic acids. The end product may be the converted substrate or increased quantities of the microbial cells (as in production of baker's yeast).

Whereas the first type of process application involves use of the microbe or enzymes in a closed, controlled environment, the second category of application, uncontained and semi-contained processes, may involve direct contact of the microbe with the environment.

Uncontained and semi-contained processes involve use of the microbe to recover a product by non-fermentation processes such as adsorbing metals from metal bearing wastes. Additionally, the microbe may be used to effect a specific physical or chemical change in the environment such as repressurizing a spent oil field through in-situ CO_2 production. Examples of such process applications include use of the organism to recover minerals from ore, to enhance oil recovery, or to clean up chemical spills and uncontrolled waste disposal sites. The use of microbial cultures in conventional wastewater treatment is thoroughly documented elsewhere and is not addressed in this report. Agricultural applications of biotechnology involve a unique set of processes to produce plants with enhanced qualities. This is a broad topic in and of itself and is better addressed independently.

The following sections of this report provide generic process descriptions for the various commercial applications of biotechnology. Sections 3.0, 4.0, and 5.0 present the processes associated with culture production, contained product manufacturing processes, and uncontained and semi-contained processes, respectively. In each of these sections, the waste streams associated with these processes are also identified. In Section 6.0 the potential impacts of these waste streams are discussed. This section addresses both the impacts of the chemical contaminants in the waste streams and the presence of the microbe as a contaminant. The effectiveness/appropriateness of current control technologies on chemical and biological contaminants is discussed and areas for which adequate data are not available are identified.

SECTION 3

CULTURE PRODUCTION

This section addresses the processes by which microbial cultures capable of producing a desired effect are identified and/or synthesized and the requirements for culture purification and scale-up.

3.1 IDENTIFICATION AND/OR SYNTHESIS OF MICROBIAL CULTURES

3.1.1 Natural Selection

Microorganisms are widespread in nature and have adapted through the process of natural selection to a diverse range of environmental conditions. Many of these organisms have been isolated into pure culture in the laboratory and have been utilized to make useful products. While the current trend seems to be more in the direction of using genetically engineered microorganisms to improve and expand existing commercial processes, the isolation of organisms from nature continues. The continued use of the natural or "wild type" organisms stems most likely from the diversity of organisms: 1) to use various waste material substrates such as corn cobs or wood pulp as nutrients to produce more useful products such as ethanol and organic acids, 2) to detoxify and degrade sewage and industrial wastes, and 3) to recover minerals and metals with less expenditure of energy resources.

The isolation of a particular microorganism from its natural environment where it lives with a mixture of other organisms is accomplished by a series of selective enrichment techniques. The purposes of these techniques are to provide a set of conditions based on pH, temperature, oxygen, nutrients, and growth factors (vitamins, enzymes) that are optimum for a particular organism at the expense of all the others. For example, Thiobacillus ferrooxidans (a bacterium important in the leaching of metals from ores) could be isolated using culture conditions which would be very hostile for most other organisms. T. ferrooxidans grow under very acidic conditions at temperatures between 20-35°C and utilize ferric iron or reduced sulfur as their energy source and carbon dioxide as their carbon source.

3.1.2 Induced Mutations

Microbial populations in nature undergo spontaneous mutation at very low rates as a result of environmental interaction or errors in the process of DNA replication. With sufficient time, money, and rapid test methods, large numbers of colonies could be screened to find organisms with the desired traits. Alternatively, mutagenic agents (mutagens) can be used in the laboratory to greatly accelerate the proportion of mutants in a controlled population. Genetic changes due to induced mutations are not predictable. While most mutagens are directed toward specific amine base sequences, it is not possible to control the site on the gene or on which gene the mutation will occur. Essentially, induced mutations result in a shotgun effect and rely on rigorous screening techniques to find the organisms with the desired traits. Genetic alterations produced in this fashion do not give the microbe any new properties that it does not already possess but merely serve to enhance existing capabilities.

Two main categories of mutagens are used: irradiation, such as X-rays and ultraviolet radiation, and chemical agents (e.g., nitroso compounds). These agents cause chemical or physical changes in the DNA structure such as:

- base pair substitutions in which a purine is exchanged for a pyrimidine (or a pyrimidine by a purine),
- addition or deletion of one or more bases (frameshift),
- base substitutions which form specific codons that cause premature initiation or termination in protein synthesis, or

- addition or deletion of longer segments of DNA within a gene which destroys gene function.

Many of these changes result in the synthesis of proteins with altered amino acid sequences which often lack their normal biological activity and can be lethal to the organism.

Application of mutagenesis has been widespread in the pharmaceutical industry to enhance production in microbial processes. Particular success has been achieved in the development of improved strains for the production of antibiotics such as penicillin and gentamycin.

3.1.3 Recombinant DNA

Recombinant DNA technology consists of the in-vitro alteration of DNA prior to insertion into a microorganism. Theoretically, DNA from any source may be introduced into a microorganism resulting in a new strain with added characteristics.

Native DNA molecules found in prokaryotic cells exist as a single, circular double strand of extreme fragility. Intact strands may be isolated from within the cells by enzymatic lysis of the cell walls followed by high-speed centrifugation or extraction with dilute salt solutions and precipitation in cold alcohol. Additional purification is accomplished by methods such as electrophoresis or chromatography.

Once separated into pure form, DNA can be broken into smaller segments by a class of bacterial enzymes called restriction endonucleases. Both strands of DNA are cleaved at specific sites (dependent on the type of enzyme used) along the chain. The separated segments possess "sticky ends" which may by hydrogen bonding reassociate to reconstruct the original break or associate with complementary strands from another source. This association can be made permanent by formation of covalent bonds by the action of DNA ligase.

After recombinant DNA fragments have been cut-and-spliced together in-vitro, a carrier or vector is needed to insert the fragment into a recipient microorganism. The plasmid is used as one such vehicle. (Plasmids are loops of self-replicating DNA which are not part of the bacterial chromosome but are readily transferred between different strains of bacteria.) The plasmid is isolated from the host cell and is cut open with a restriction endonuclease at one site. A DNA fragment is inserted between the cut ends of the plasmid and the plasmid is returned to its normal circular form. Once reassembled, the recombined plasmid can be introduced into a microorganism where it is replicated and inherited as part of the microorganism.

3.1.4 Cell Fusion Methods

Cell fusion consists of fusing two separate cells into a single unit which is then endowed with the properties from both cells. In general, two approaches are used: monoclonal antibodies (hybridoma techniques) and protoplast fusions.

Monoclonal Autobodies--

Monoclonal antibodies are produced by injecting a test animal (usually a mouse or rat) with an antigen or target chemical. The immune system of the test animal responds and begins to proliferate antibody-forming cells (lymphocytes). After a few days the spleen (rich in lymphocytes) is removed from the test animal. The lymphocytes are mixed with myeloma cells (a special type of lymphocyte tumor cell) and are fused into hybrid cells (hybridomas) by treatment with polyethylene glycol. The hybridoma has both the capacity to produce antibody from the parent lymphocyte parent and the capacity to proliferate indefinitely from the myeloma parent.

After fusion, the antibody-forming hybrid cells are separated from a mixture which also contains unfused lymphocytes and myeloma cells. The hybridoma cells are grown in tissue culture and the desired clones are identified by suitable selection procedures based on the original material injected into the test animal.

The initial development of the hybridoma technique and commercial applications of the process were produced in cells from laboratory mice. More recent developments have extended the use with human lymphocytes. At the present time, the major monoclonal antibody product categories are: research reagents, clinical diagnostic kits, agricultural products, and industrial applications (affinity separation agents).

Protoplast Fusion--

Protoplast fusion techniques generally are applied to microorganisms or plant cells which have tough outer cell walls that surround and protect a thinner cell membrane. The cell wall is dissolved using enzymes, and a spherical entity called a protoplast remains. Because cell membranes have similar composition in most species, protoplasts of different species can be induced to fuse by several means. The hybrid cells formed contain genetic material from both contributing cells.

In the past, protoplast fusion techniques have been used on bacteria and fungi primarily to improve yields of antibiotics and organic acids. More recent applications have allowed researchers to grow several species of plants in tissue culture from single cells to mature multicellular form.

3.2 SELECTION/PURIFICATION

Isolation of the microbe or cell type desired from a large mixed microbial population generally involves the use of a trait or a series of traits which is unique to the particular organism or cell. This may be accomplished by using nutritional requirements, antibiotic resistance, growth rates, cell markers, or other novel cell sorting techniques.

In some cases the isolation procedures are straightforward and simply involve designing a controlled growth environment which is very selective for the desired cells. An essential nutrient which can be synthesized only by the mutant organism can be deleted from the growth medium so that only the mutants are able to grow. Mutants with antibiotic or chemical resistance can be selected by culturing numerous cells of the starting strain on plates containing the antibiotic or inhibitory chemical. Only the resistant cells survive and form colonies.

Sensitive strains in an otherwise resistant population are somewhat harder to isolate and have in the past required that very large numbers of individual colonies be screened for the desired property. With conventional techniques such as replica plating, the process is very laborious and even the best equipped labs are unable to screen more than 500 colonies per week. New automated techniques have been developed commercially which have vastly increased screening capacity. Researchers have developed a system that uses an automated scanner to scan 2-3 million colonies cultured in regular arrays on numerous large glass trays covered with solid nutrient media. The desirable colony characteristics are programmed into a computer and any colonies which show these characteristics are retained automatically by transfer to a holding medium for later processing.[1]

3.3 SCALE-UP

Once the biological agent has been selected and isolated into a pure culture it is necessary to increase the quantity of the culture from experimental or laboratory scale to industrial scale. At the same time great caution must be exercised to preserve the purity, viability, and desired traits of the original selected culture. Spore and vegetative suspensions, slants and roux bottles, surface colonies, and lyophils are a few examples of methods used for stock culture maintenance.

Submerged cultures are the predominant techniques used for culture propagation. Generally, a flask of liquid medium is inoculated with a lyophilized or frozen culture and incubated until sufficient cell mass has been produced. Once the culture has increased, the flask is used to inoculate a seed fermenter. In the seed fermenter, the inoculum is serially scaled up in stages. The number of stages or scale-ups necessary is somewhat dependent on the industry. Based on the assumption that transferring and media sterilization are the main contamination routes, the main objective is to have the fewest possible transfers from the primary stock culture to the end use.

3.4 WASTE STREAMS ASSOCIATED WITH CULTURE PRODUCTION

Waste streams associated with culture production generally are comparable to those associated with pharmaceutical research. Major sources of wastewater are equipment and vessel washing, animal cage wash water, and spent broths from laboratory-scale production units. In general, quantities are relatively small in relation to those from a production facility and are characterized as having BOD and COD concentrations similar to those in domestic sewage. These wastes would also be expected to contain viable microorganisms. The materials discharged frequently vary in both quality and quantity and may be released on a discontinuous basis.

Flammable solvents, especially volatile solvents such as ethyl ether, are considered the most common problems in pharmaceutical research.[2] Additional problems include infectious wastes containing residues of the microorganisms and test animal carcasses.

Aqueous wastes from culture production in the pharmaceutical industry are often discharged into a publicly owned treatment works (POTW). NIH guidelines may require sterilization of wastes prior to discharge if the waste is likely to contain any genetically altered microorganisms. Solid wastes, liquid wastes, and aerosols which are potentially infectious in nature must undergo treatment that will ensure destruction of pathogenic agents. Autoclaving and incineration are the two methods most commonly used in the pharmaceutical industry.[2]

Test animal carcasses and other disposal materials are steam sterilized, incinerated, and/or landfilled depending on whether they are contaminated with an infectious agent.[2]

SECTION 4

CONTAINED PRODUCT MANUFACTURING PROCESSES

A major application of microbial cells and enzymes is in the conversion of feedstock materials (substrates) to useful products. This type of application is analogous to in-plant chemical processing and in some cases is in direct competition with traditional chemical synthesis.

Contained product manufacturing processes are defined as fermentation processes which employ microbial cultures or enzymes in a closed vessel for the conversion of an organic substrate material to a useful product. There are three major process components in product manufacturing processes: substrate preparation, fermentation, and product recovery and purification. Substrate preparation involves pretreatment of the feedstock material to convert it to a form suitable for microbial conversion. In fermentation the prepared substrate is contacted with the microbe and converted to the product compound through enzymatic reactions. Alternatively, the substrate is utilized purely as a food source to increase the rate of cell growth and the cells themselves are harvested as the product. The final process involves recovery of the product from the fermentation broth through a variety of separations technologies.

The application of fermentation technology for product production is by no means new. This technology has been employed in the production of alcoholic beverages, antibiotics, and foodstuffs for many years. However, recent advances in genetic engineering and fermentation technology have widened the spectrum of potential product applications. Additionally, the potential for use of non-petroleum based renewable substrates also has served to advance research in this industry.

Fermentation technology can be used to produce fuels, chemical feedstocks, enzymes, food additives, animal feeds, specialty chemicals, and numerous medicinal products. Millis has grouped the major chemicals produced by microorganisms by their method of microbial conversion.[3] These include:

End products of normal metabolism - alcohols
solvents
acids
methane
biomass,

Primary metabolites of biosynthesis - amino acids
nucleotides
vitamins
enzymes,

Secondary metabolites - antibiotics
polymers
pigments
surfactants
gibberellins, and

Transformation products - steroids
alkaloids
hydrocarbons
terpenes
antibiotics.

End products of normal metabolism are residual compounds produced by the microbe as a result of its growth and reproduction. Primary metabolites are intermediate compounds synthesized from the substrate by the microorganisms for its subsequent use in growth and reproductive processes. Secondary metabolites are synthesized by the microbe after growth ceases and are not involved in growth or reproduction. Transformation products are compounds formed due to the catalyst action of the microbe. A specific site on a complex molecule can be changed due to oxidation, reduction, hydroxylation, or dehalogenation, etc. These compounds do not necessarily contribute directly to the growth of an organism.

Products currently manufactured by fermentation processes on an industrial scale include organic acids such as acetic, citric, and lactic acid; ethanol; animal feed (single cell protein); enzymes; fructose; food additives; and medicinals. The extent to which fermentation technology is more widely applied will, in most cases, be dependent on process economics. In general, fermentation processes are in direct competition with conventional petroleum-based chemical processes. As such, the production of fuels and tonnage commodity chemicals such as ethanol are not currently considered economically viable. The most rapid growth in the application of fermentation technology can be expected in the production of specialty chemicals and enzymes for which there is no chemical processing equivalent and in processes which utilize inexpensive unrefined substrates as a feedstock.[4] Continued use of fermentation technology can be expected in the production of medicinals because, for these products, cost is typically considered secondary to need and effectiveness by the user.[4]

The following sections present process descriptions for each of the three major process units in fermentation technology: substrate preparation, fermentation, and product recovery and purification. Additionally, the waste streams associated with each of these process units are identified.

4.1 SUBSTRATE PREPARATION

Substrate preparation typically consists of: pretreatment to convert the substrate into a form suitable for fermentation, sterilization, nutrient addition, and pH adjustment. The extent of required substrate pretreatment is dependent on the type of substrate employed. Similarly, the degree and type of sterilization methods, nutrient addition, and pH adjustment will vary with specific process requirements. Figure 6 presents a generalized flow diagram of substrate preparation processes.

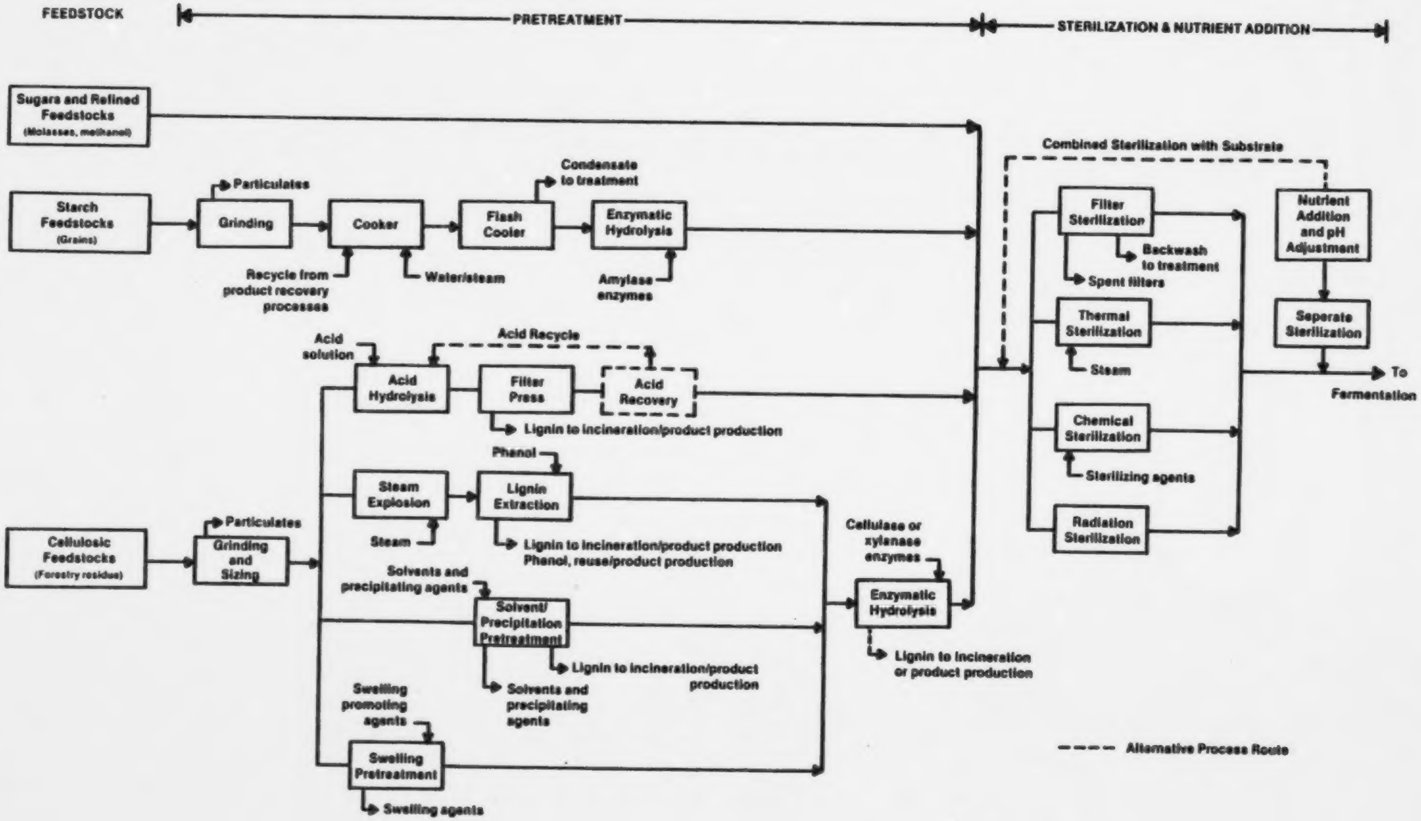

FEEDSTOCK
PRETREATMENT
STERILIZATION & NUTRIENT ADDITION
Sugars and Refined Feedstocks (Molasses, methanol)
Starch Feedstocks (Grains)
Grinding
Particulates
Cooker
Recycle from product recovery processes
Water/steam
Flash Cooler
Condensate to treatment
Enzymatic Hydrolysis
Amylase enzymes
Cellulosic Feedstocks (Forestry residue)
Grinding and Sizing
Particulates
Acid solution
Acid Hydrolysis
Acid Recycle
Filter Press
Acid Recovery
Lignin to incineration/product production
Steam Explosion
Steam
Phenol
Lignin Extraction
Lignin to incineration/product production
Phenol, reuse/product production
Solvents and precipitating agents
Solvent/ Precipitation Pretreatment
Lignin to incineration/product production
Solvents and precipitating agents
Swelling promoting agents
Swelling Pretreatment
Swelling agents
Cellulase or xylanase enzymes
Enzymatic Hydrolysis
Lignin to incineration or product production
Combined Sterilization with Substrate
Filter Sterilization
Backwash to treatment
Spent filters
Thermal Sterilization
Steam
Chemical Sterilization
Sterilizing agents
Radiation Sterilization
Nutrient Addition and pH Adjustment
Separate Sterilization
To Fermentation
Alternative Process Route

Figure 6. Substrate Preparation Processes.

4.1.1 Process Description

Substrate Pretreatment--

The type of substrates employed in fermentation processes can be grouped into three categories according to the nature of their carbohydrate content. These include sugar and refined feedstocks, starches, and cellulosic substrates.[5] The first group, sugars and refined feedstocks, consist of substrates such as molasses, fruit extracts, coconut milk, and conventional chemical feedstocks such as methanol. These substrates typically do not require any pretreatment prior to sterilization and subsequent fermentation.

Starch substrates consist primarily of food crops such as cereals, rice, and cassava. These substrates require significant pretreatment to convert the starches to sugars which can be utilized by the microbial culture. The raw feedstock is first passed through a grinding operation and may undergo treatment to remove protein, oils, and fiber. The substrate is then diluted with water and potentially with recycled streams from downstream processes. The mixture enters a cooker where the starch is solubilized at elevated temperatures of approximately 135°C (275°F).[6] The solubilized starch ("mash") is typically converted to sugars by enzymatic hydrolysis with amylase from bacteria or filamentous fungi. Although chemical hydrolysis may be used, at present enzymatic hydrolysis is preferred.[5] The use of amylase is a notable difference from conventional beverage fermentation plants; both federal regulation and final product flavor require distillers to use an enzyme derived from malt.[6] After cooking, the "mash" is flash cooled and, depending on product needs, may be passed directly to the fermenter or further prepared by sterilization and nutrient addition. It should be noted that specific pretreatment operating conditions will vary with fluctuations in feedstock source, purity and quality.

Cellulosic substrates consist of agricultural and forestry residues such as bagasse and wood remains and require fairly expensive physical/chemical pretreatment. The major components of cellulosic substrates are cellulose, hemicellulose, and lignin. The composition of various substrates varies; however, most materials contain 15-25 percent hemicellulose, 30-45 percent cellulose, and 5-15 percent lignin.[7] Actual compositions of various substrates are presented in Table 4.[8]

Only the cellulose and hemicellulose fractions are available as carbohydrate for conversion by biological methods. The cellulosic fraction consists almost entirely of glucan[7] and in nature exists as a fibrous crystal.[9] Hemicellulose consists of varying quantities of glucan and xylan.[7] The lignin fraction consists of phenolic polymers and is difficult to degrade into individual chemical components.[9,8] In the plant tissue lignin functions as a cement between individual fibers and as a protective sheath against microorganisms.

Thus, the pretreatment of cellulosic substrates involves freeing the cellulosic material from the lignin matrix and conversion of the cellulose and hemicellulose to sugars. The technologies to enhance this conversion are currently the subject of active research and development.

The primary method of pretreatment of cellulosic substrates for biological conversion is acid hydrolysis. The raw feedstock is first passed through grinding and sizing operations and can then proceed directly to acid hydrolysis where the cellulose and hemicellulose are solubilized and converted to sugars.[10] The hydrolysis can be accomplished at high temperature with dilute acid solutions or at low temperatures with concentrated acid. In the latter process, the sugars are separated from solution and the acid recovered for reuse. In dilute acid processes there is no need for acid recovery, and the solution is neutralized with a suitable base prior to fermentation.[7] The lignin remains in the solid phase and is separated by filter presses. The lignin residue can be converted to useful products; however, the value of the lignin for reuse is somewhat diminished due to the sulfonation and crosslinking which occurs during the acid hydrolysis process.[10]

TABLE 4. TYPICAL CHEMICAL COMPOSITION OF CELLULOSIC SUBSTRATES

Biomass Substrate/Feedstock	Chemical Components (%)		
	Cellulose	Hemicellulose	Lignin
Hardwoods	45	30	20
Softwoods	42	27	28
Wheat Straw	30	27	11
Corn Stover	38	26	19
Hemlock Bark	26	16	15
Feedlot Manures	17	19	7
Municipal Solid Waste	61	NA	NA
Domestic Sewage Sludge (dry)	35	6	NA

NA = Not Available

Reference: 8.

During acid hydrolysis, the xylose and glucose produced may further degrade to furfurals which are toxic to microorganisms.[7] Shah, et al. describe a two-stage acid hydrolysis system that first hydrolyzes the hemicellulose at mild conditions.[7] After separation the solids are again hydrolyzed in the second phase at much more severe conditions to hydrolyze the cellulose fraction. This process reduces the potential for further degradation of the sugars. The remaining solids consist of unconverted cellulose and lignin residue.

Cellulosic substrates may also be hydrolyzed enzymatically; however, this process requires physical or chemical pretreatment to separate the cellulose and hemicellulose from the lignin and to disrupt the crystalline structure of the cellulose. The most widely used physical pretreatment is steam compression/decompression or "steam explosion."[10] The steam explosion plasticises the lignin both freeing the cellulosic material and reducing its crystallinity. The lignin may subsequently be removed by phenol extraction. This separation process yields a relatively pure lignin which may be further processed to useful products such as phenolic resins.[10]

The freed cellulose fractions can then be converted to sugars via enzymatic hydrolysis with cellulase or xylanase enzymes.[7] Future areas of research may address methods of simultaneous enzyme hydrolysis and fermentation.

Alternatively, the substrate may undergo solvent extraction or swelling pretreatment. In solvent extraction pretreatment, the crystalline structure of cellulose is destroyed by dissolving it in solvent with subsequent reprecipitation of the cellulose. Potential solvents and precipitating agents include phosphoric acid, cadoxen, hydrazine, sulfuric acid, ferric tartrate, and cuprammonium sulfate.[9,8] No information was found on the fate of lignin in this process, however. It is felt that the lignin is separated from the solvent/cellulose mixture.

Swelling pretreatment requires addition of a chemical reagent which results in intracrystalline swelling thus rendering the cellulose more reactive to enzymatic hydrolysis. Potential swelling agents include sodium hydroxide, organic bases such as ethylenediamine, and some salts.[9] In this process the lignin remains in the substrate. It is reported that the presence of lignin may not interfere with the enzymatic hydrolysis.[9] However, the presence of lignin may be toxic to microorganisms in the subsequent fermentation process.[8] Therefore, the lignin fraction is most probably removed after conversion of the cellulosic fractions.

Sterilization--

In most fermentation processes there is a need to maintain aseptic conditions as most products are produced by a pure culture. If foreign cultures contaminate the process they may inhibit or destroy the biological catalyst, destroy the product, or introduce noxious substances that are difficult to separate from the product.[11] Additionally, the microbial culture may become infested with a phage, a naturally occurring virus that attacks microorganisms.[2] To prevent such contaminations, all materials entering the fermentation vessel are sterilized and subsequently transferred to the vessel aseptically. Alternatively, the materials may be charged to the vessel prior to introduction of the culture, and the vessel and substrate may be sterilized simultaneously.[12]

Technical problems may arise with sterilization as the available methods may interfere with the stability of the substrate.[13] Additionally, maintaining the aseptic integrity of entry and exit points is extremely difficult and serious losses due to contamination are not uncommon.[11]

For clear solutions free of suspended solids, the substrate may be sterilized by absolute filters with a pore size small enough to trap any organism or virus particle in the liquid. Such filters include unglazed ceramic and plastic membranes and granular precoat filters.[12] The substrate may first be passed through coarse granular or fiber filters to prevent "blinding," or clogging, of the sterilization filter. One problem associated with sterile filtration is the reduction in flow rate due to the reduced pore size; treatment of the substrate at industrial flow rates requires very large filter surface areas.[12]

Complex substrates which contain insoluble materials may be sterilized by heat treatment.[13] This may be accomplished by simply heating the substrate to a temperature high enough to kill living organisms and holding for a period adequate to achieve sterility or by direct steam sparging of the liquid. Steam sparging is very rapid but may produce severe foaming. The steam must be free of anti-corrosion additives and dilution by the steam condensate must be accounted for in process material balances.[12] In direct heating processes the substrate is subsequently cooled in heat exchangers or flash evaporators. Flash evaporation provides very rapid cooling; however, this process may produce severe foaming and water losses in the condensate must also be accounted for.[12]

Heat sterilization may not be appropriate for many substrates as some components lose stability or react at sterilization temperatures. Alternative methods of sterilization include radiation or treatment with chemical sterilizing agents such as ethylene oxide.[12]

Nutrient Addition and pH Adjustment--

In general, the raw feedstock will not contain all of the necessary nutrients and trace elements required by the microbial culture.[5] The primary nutrient requirements are sources of nitrogen and phosphorus such as ammonium, nitrate and phosphate salts. Inorganic compounds such as sulphates, sodium and potassium salts, trace elements, and vitamins may also be required; and, depending on the substrate and pretreatment processes, chemical addition for pH adjustment may be necessary.[13]

These additives may be charged to the substrate prior to sterilization or sterilized separately by the methods previously discussed.

4.1.2 Waste Streams from Substrate Preparation

Table 5 presents a summary of the potential waste streams from substrate pretreatment processes and sterilization processes identified in the literature for the three categories of substrates. The table also presents current treatment and disposal practices for these wastes where such data are available. In general, most fermentation facilities employ centralized biological wastewater treatment facilities with pretreatment of isolated waste streams for specific pollutant removal as necessary.[2] It should be noted that this presentation does not include waste streams from ancillary operations such as cooling tower blowdown and wastes associated with steam and energy production, as these wastes are not unique to fermentation processes.

The only waste stream identified in starch substrate pretreatment processes is the condensate from the flash cooler. This is a fairly innocuous waste stream comprised entirely of water and contributes only low levels of BOD and solids to wastewater treatment.[6]

A major residual from preparation of cellulosic substrates is the lignin residue. In the acid hydrolysis process this residue is recovered as a filter cake and may be used for subsequent conversion to useful products such as phenolic resins, chelating agents, and emulsion stabilizers.[14] However, the acid hydrolysis process produces a lower quality lignin for such conversions, and this residue is often incinerated as it is reported to be difficult to treat.[10,4]

In enzymatic hydrolysis processes the lignin may be chemically extracted from the substrate following steam explosion. This process produces a higher quality lignin for potential use. No mention of lignin residues from solvent/precipitation and swelling processes was found in the literature; however, it is suspected that at some point the lignin is separated from the substrate either during the pretreatment process or following enzymatic hydrolysis.

TABLE 5. WASTE STREAMS FROM SUBSTRATE PREPARATION

Process Unit	Waste Stream	Potential Contaminants	Current Treatment/ Disposal Methods
PRETREATMENT			
Sugars and Refined Substrates	no waste stream identified	-	-
Starch Substrates			
Grinding	particulate emissions	dust	cyclones, baghouses
Flash Cooler	condensate/water	BOD $\sim$ 10 mg/L TS $\sim$ 80 mg/L TSS $\sim$ 10 mg/L	conventional biological wastewater treatment
Cellulosic Substrates			
Grinding	particulate emissions	dust	cyclones, baghouses
Acid Hydrolysis	filter cake/lignin, ash	polymers aromatics	incineration used in production of products
Enzyme Hydrolysis			
steam explosion	No data	No data	No data
lignin extraction	lignin residue	spent solvent phenols polymers aromatics	used in production of products incineration
solvent extraction/precipitation	spent solvent and precipitating agents	phosphoric acid cadoxen hydrazine sulfuric acid ferric tartrate cuprammonium sulfate	No data
	lignin	polymers aromatics	used in production of products incineration
swelling pretreatment	swelling agents	sodium hydroxide organic bases (amines) salts	No data
	lignin	polymers aromatics	used in production of products incineration
STERILIZATION			
Filtration	spent filters	viable microorganisms feedstock residual	incineration landfill
	backwash	viable microorganisms high solids feedstock residual	conventional biological wastewater treatment
Thermal, Chemical, Radiation	No data	No data	No data

Solvent extraction/precipitation pretreatment could produce waste streams containing spent solvent and precipitating agents, some of which may be toxic (e.g., cadoxen and hydrazine). However, solvent recovery processes are most likely employed to improve the economics of this process. Similarly, swelling pretreatment waste streams will contain swelling agents such as sodium hydroxide, organic bases, and salts. No discussion was found as to the potential recycle of these streams.

Sterilization by filtration results in a residual of spent filters and most probably produces a liquid waste stream from backwash operations. This waste stream probably would be treated in centralized wastewater treatment facilities. No data were found on waste streams associated with thermal, chemical, or radiation sterilization of substrates.

4.2 FERMENTATION PROCESSES

In the fermentation process the substrate is converted to structurally related compounds in an aerobic or anaerobic environment by one or more enzymes supplied by microbial cells. The converted substrate may be the desired product or the cells themselves may be harvested with the substrate serving to enhance cell growth. Products may be formed both intracellularly or, more desirably, excreted through the cell membrane. In the former case, the cells must be disrupted prior to product recovery processes.

Fermentation can be accomplished either by addition of the enzymes or by addition of the microbial cell which produces the enzyme. An advantage of employing the enzyme alone is the high degree of specificity characteristic of enzymatic reactions. This reduces the possibility of side reactions and increases product yields. The microbial cell, on the other hand, may produce hundreds of different enzymes not all related to the production of a single product.[1] However, most biological conversions require a succession of interlocking reactions, each catalyzed by a different enzyme thus requiring the use of the entire microbial culture.[11] An active area of research is the development of improved microbial cells for biological conversions through recombinant DNA and mutations engineering.

Fermentation processes may be accomplished by submerged or immobilized processes. In the former, the culture or enzyme is completely mixed and maintained in suspension with the substrate. In immobilized processes the culture or enzyme is supported or encapsulated in a nutritionally inert matrix and the substrate is passed over the fixed structure. Figure 7 presents a schematic of the basic fermentation processes. Traditional fermentations are operated with submerged cultures in batch processes. However, recent advances have been made in the development of continuous fermentation processes. The use of immobilized cultures and enzymes represents the newest process research to adapt fermentation technology to industrial scale.

4.2.1 Process Descriptions

Submerged Fermentation Processes--

As previously stated, traditional submerged fermentations are operated in a batch mode. Once the substrate is in a form suitable for biological conversion it is then charged to the fermenter aseptically and inoculated with the appropriate microbial culture or enzyme. The culture is maintained in suspension in the substrate and allowed to incubate for periods ranging from a few hours to several days.[15] Gaseous products of fermentation are continually vented from the fermenter. After the conversion is completed, the entire contents of the fermenter are withdrawn and passed to product recovery and purification processes, and the fermenter is prepared for the next batch.

The fermentation vessel must provide a controlled and protected environment for the cell, necessitating rigorous control of sterility, mixing, oxygen and nutrient requirements, foaming, pH, temperature, and pressure.

Prior to each batch the fermenter must be sterilized by methods similar to those discussed under substrate sterilization. Maintaining aseptic conditions is highly important as the introduction of any contaminants may result in the dumping of entire fermentation batches. The most common method of vessel sterilization is direct wet steam sparging. Prior to steam sparing the vessel must be thoroughly cleaned and all air removed as the presence of a

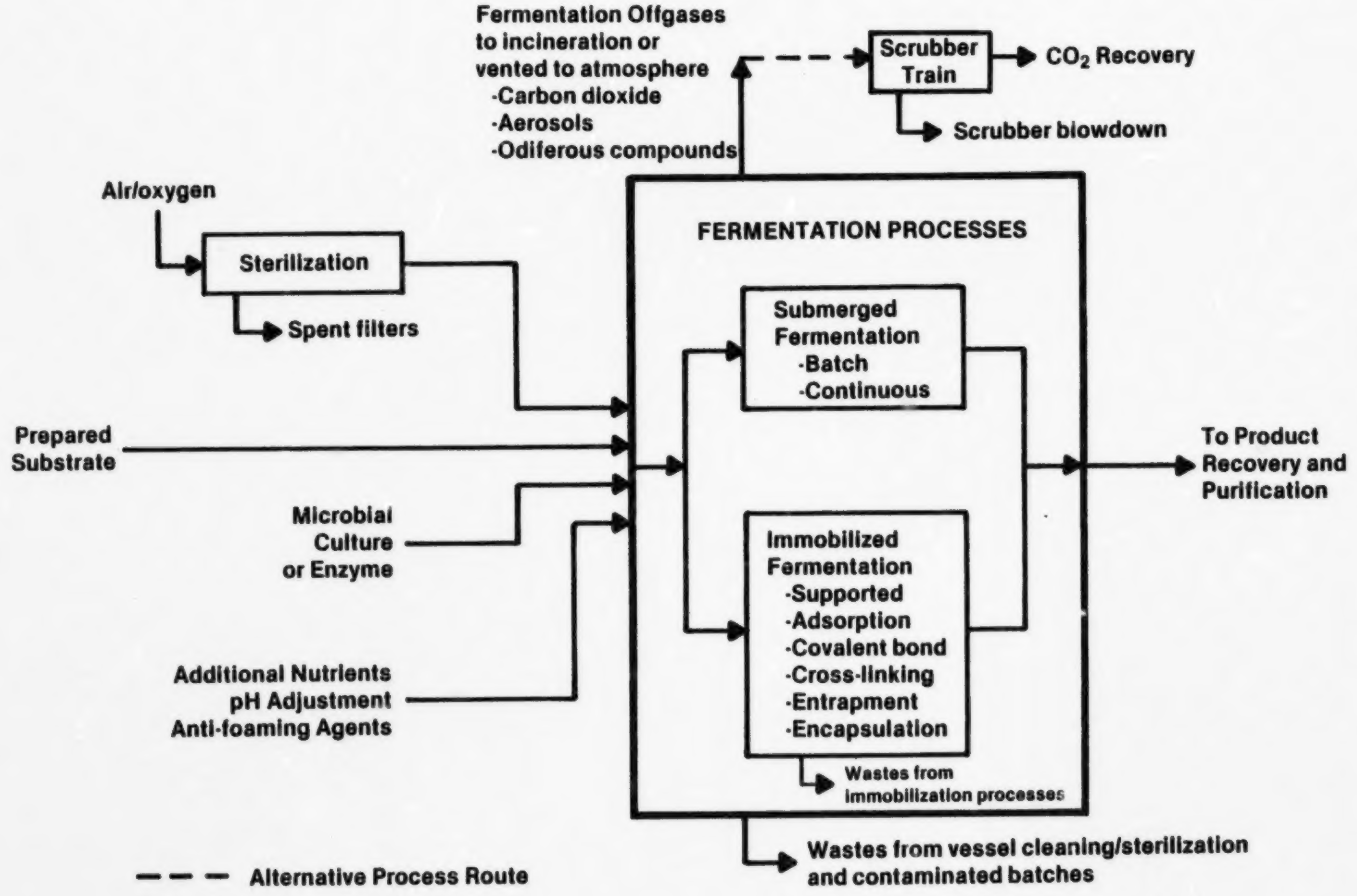

Fermentation Offgases
to incineration or
vented to atmosphere
-Carbon dioxide
-Aerosols
-Odiferous compounds
Scrubber Train
CO_2 Recovery
Scrubber blowdown
Air/oxygen
Sterilization
Spent filters
Prepared Substrate
Microbial Culture or Enzyme
Additional Nutrients
pH Adjustment
Anti-foaming Agents
FERMENTATION PROCESSES
Submerged Fermentation
-Batch
-Continuous
Immobilized Fermentation
-Supported
-Adsorption
-Covalent bond
-Cross-linking
-Entrapment
-Encapsulation
Wastes from immobilization processes
To Product Recovery and Purification
Wastes from vessel cleaning/sterilization and contaminated batches
Alternative Process Route

Figure 7. Fermentation Processes.

dirt layer or air pocket may prevent contact of the steam with the vessel surface.[12]

Chemical sterilization may also be used for plant components which may be damaged by high temperatures such as pH probes and sensors. The components first must be cleaned to ensure contact with the biocidal agent and any residuals must be removed after treatment by washing with sterile water.[12] Fumigation with gaseous or volatile biocides such as ozone, formaldehyde, sulfur dioxide, or ethylene oxide also may be used; however, some of these agents may leave a coating on components which is difficult to remove.[12]

Adequate mixing must be maintained in submerged culture fermentations to ensure contact of the substrate with the microbial culture. In a continuous stirred tank reactor (CSTR), mixing is accomplished by a central rotating impeller vane. This process presents some technological problems in that such mixing may have little effect at the micrometer scale of the cells and increasing impeller speed may create shear forces damaging to the microbe. Additionally, the point of entry of the drive shaft creates a leak path for contamination.[15] Alternatives to the CSTR include the use of air lift fermenters, in which the addition of compressed air provides mixing, and the use of loop reactors.[15,13]

In aerobic fermentation processes the mixture is aerated throughout the incubation period. The most common source of supply is filtered air; however, oxygen enriched air from cryogenic fractionation also may be used.[11] A major problem associated with fermentation processes is the production of aerosols. The aerosols escape the fermenter with the exhaust gases and may transport enzymes or microbes harmful to plant workers. This release could be of even more concern if genetically modified microbes are used in industrial processes, not only in terms of worker safety but also due to release of such microbes to the environment.

Additional nutrients, chemicals for pH adjustment, and anti-foaming agents may be added as required during the fermentation process. Temperature is controlled by heating/cooling coils in the reactor or by water jackets that surround the vessel.

The advantage of batch fermentation is that the systems are easily built and operated, and if desired a different product can be produced in each batch. This would be desirable for products which are produced in small quantities, such as medicinals. Batch processes do, however, require a significant amount of time between batches for vessel preparation. One solution to eliminate reactor downtime is the semicontinuous process. In this process a residual of culture and broth is left in the reactor to serve as inoculate for the next batch of the same product and sterilization between batches is eliminated. However, the potential for contamination is increased.

Continuous fermentation could potentially have many advantages over batch processes, such as increased production rates and higher product yield. In batch fermentation high substrate concentrations during the initial phase and high product concentrations in the final stages often inhibit microbial growth. Continuous processes, however, can operate at the optimal stage of microbial growth or in some cases at a stage where growth does not occur but conversion does.[5,15]

Continuous fermentation reactors are essentially batch reactors with inlet and outlet ports for the continuous addition of substrate and nutrients and the continuous withdrawal of broth and cells. The conversion reaction can be maintained at steady state by limiting cell growth through nutrient addition to exactly balance cell loss.[15] It should be noted, however, that continuous fermentation processes are more prone to contamination.

Immobilized Fermentation Processes--

The use of immobilized cells or enzymes may provide the optimal process for continuous fermentations in which the product is produced extracellularly.

In these fermentation systems the enzyme or microbe is immobilized in or on an inert matrix, and the substrate is converted as it passes over the solid matrix.

The advantages of immobilization processes include:

- recovery and reuse of the cell or enzyme,
- increased stability,
- elimination of cell separation from product recovery processes,
- rapid termination of reactions, and
- protection of the cell or enzyme from denaturation.

The major problem associated with immobilization processes is ensuring adequate contact with the substrate. Additional problems include loss of the biocatalyst from the support matrix and inactivation of the cell or enzyme by the immobilization process.

Microbial cells can be immobilized by supporting the cell on a solid matrix or by more complex procedures such as adsorption, covalent bonding, entrapment in polymeric gels, cross-linking, and encapsulation. In general, research on immobilizing enzymes is more advanced than cell immobilization technology. However, interest in processes to immobilize cells for conversions requiring multi-enzyme reactions has increased steadily in the last ten years.[16] Additionally, most of the methods used to immobilize enzymes can also be used for microbial cells. Some of the advantages of immobilized cells over enzymes include:[8]

- increased biocatalytic activity,
- elimination of enzyme extraction and purification processes,
- greater operational stability,
- the ability to employ intracellular or unstable enzymes, and
- the ability to effect multi-enzymatic reactions.

Table 6 summarizes the current techniques used for immobilization of cells and enzymes as well as the advantages and disadvantages associated with each. The oldest and most common technique for immobilization of cells employs a packed bed consisting of a solid support on which the cells are encouraged to grow. This techinque, which is analogous to the trickling filter in wastewater treatment, is currently employed in the production of acetic acid.[11]

Other support type immobilization processes include adsorption and covalent bonding. Adsorption processes are probably the mildest immobilization methods available.[12] In adsorption processes an enzyme or cell solution is added to the support, the system stirred, and unattached enzymes or cells washed away. The most popular supports are ion-exchange resins.[17] Other supports include alumina, glass, silica gel, collagen derivatives, kaolin, and polystyrene resins.[1] This process is most suited for cell immobilization. Conversely, covalent attachment to chemically active supports is more suited to enzymes. The most common support used in this process is porous ceramics although nylon tubes, acrylic polymers, and cellulose derivatives are also used.[17,1]

Alternative methods for cell and enzyme immobilization involve cross-linking, entrapment, and encapsulation. Cross-linking is more suited for enzymes and involves linking the enzyme with bifunctional agents to precipitate agglomerates of enzymes or to chemically incorporate the enzyme in a polymer.[17]

Entrapment in polymeric gels is one of the most widely applied methods of immobilization and is especially good for cells. In this process the cells are not directly attached to the support surface but rather the cells are added to a solution of monomer before polymerization, resulting in entrapment of the cell in the polymeric matrix.[17] The most common methods use polyacrylamide, collagen, cellulose acetate, calcium alginate, or carrageenan as the support matrices.[12]

TABLE 6. TECHNIQUES FOR IMMOBILIZATION OF MICROBIAL CELLS AND ENZYMES

Immobilization Process	Advantages	Disadvantages
ADSORPTION ion exchange resins alumina glass silica gel collagen derivatives kadin polystyrene resins	most suited for cell immobilization; simplest method; mild conditions, no damage to cells; high activity; inexpensive	unstable product; leakage of cells
COVALENT BONDING porous ceramics nylon tubes acrylic polymers cellulose derivatives	most suited for enzymes; simple process; durable; may increase enzyme activity	potential inactivation of enzymes
CROSS-LINKING	most suited for enzymes; stable attachment	difficult process; attachment may destroy enzymatic activity; may not be able to achieve substrate contact with the enzyme
ENTRAPMENT polyacrylamide collagen cellulose acetate calcium alginate carrageenan	especially good for cells; mild conditions; long life; inexpensive	may not be able to achieve substrate contact with the cells; enzyme inactivation
ENCAPSULATION microcapsules liposomes hollow fibers dialysis membranes	highly stable immobilization; gentle process; substrate can easily contact biocatalyst	very expensive; no stabilization of activity

References: 17, 8, 18.

The final immobilization process involves encapsulation of the cells or enzymes in microcapsules, liposomes, hollow fibers, or dialysis membranes. While this process is highly effective it is also very expensive and most suited for production of medicinals.[17]

4.2.2 Waste Streams from Fermentation Processes

There are relatively few waste streams associated with the fermentation process itself. Most of the high strength, high volume liquid and solid waste streams are produced in subsequent product recovery and purification processes. A summary of the waste streams associated with fermentation is presented in Table 7. The only continuous waste stream emitted during the actual fermentation process is the waste offgases. The air and gas vented from the fermenter contain large amounts of carbon dioxide or methane and may contain aerosols, residual product, viable microbes, and odiferous substances.[2] In aerobic processes, this stream is often vented directly to the atmosphere; however, treatment by filtration, incineration, or scrubbing may be necessary to contain microorganisms and to deodorize the gas.[2] Additionally, by-product carbon dioxide can be recovered through a scrubber train.[6] The blowdown from scrubbers may contain absorption chemicals, light soluble organic compounds, and heavier insoluble organic oils and waxes.[2] In anaerobic processes, the methane produced may be flared or recovered as a by-product.

Large amounts of liquid wastes may be generated from vessel sterilization and the dumping of contaminated or "off-spec" fermentation batches. Sterilization wastes may contain detergents, biocides, and viable microorganisms as well as residual feedstock, product, and intermediates. The dumping of bad batches can result in very large wastewater discharges in a short period of time. These wastes are characterized by high BOD, COD, and TSS concentrations and will contain the process microbe and/or contaminant microbes, viruses, feedstock, product, product intermediates, color, and nutrients. As discussed in substrate pretreatment, these wastes are most often treated in centralized biological wastewater treatment facilities.

TABLE 7. WASTE STREAMS FROM FERMENTATION PROCESSES

Fermentation Process	Waste Streams	Potential Contaminants	Current Treatment/ Disposal Methods
AIR/OXYGEN STERILIZATION	spent filters	particulates and microbes found in ambient air	no data
FERMENTATION	offgases	carbon dioxide methane aerosols volatilized product microbe/enzymes odiferous compounds	vented to atmosphere scrubbers CO_2 recovery methane recovery incineration
	offgas filtration	spent filters viable microorganisms	no data
	scrubber blowdown	absorption chemicals light soluble organics organic oils and waxes	conventional biological wastewater treatment
	vessel cleaning wastes	detergents biocides viable microbes/enzymes feedstock residue product residue intermediate product residue nutrients	conventional biological wastewater treatment
	contaminated batches	high BOD, COD, TSS viable microbes/enzymes microbial virus feedstock product intermediate products nutrients	conventional biological wastewater treatment
IMMOBILIZATION PROCESSES			
adsorption	solution wash	no data (enzymes)	no data
covalent bonding cross-linking entrapment encapsulation	spent microbe/support matrix	microbes/enzymes polymers gels other supports	no data

Another source of waste associated with fermentation are those waste streams produced from the process of immobilizing cells and enzymes. Very little data were found on the specifics of these techniques; however, it is conjectured that they may produce waste streams containing a broad spectrum of chemical agents and polymers used in the immobilization process. Additionally, as the activity of the microbe or enzyme in the solid matrix will eventually decrease, the entire matrix may require periodic disposal.

4.3 PRODUCT RECOVERY AND PURIFICATION

Once the desired product(s) has been synthesized, some means must be employed for product removal from the system. Removal may be a continuous or batch process and usually involves several steps for separation and purification. If the product is produced outside the microorganism, the microbial population is first removed, and then the broth is processed. If, on the other hand, the product is produced intracellularly, the microbal population is retained for processing, and culture liquids are discarded or recycled.

The major techniques used for product isolation and purification and the major waste streams which may be associated with these techniques are presented in Figure 8 and are discussed in the following subsections.

4.3.1 Techniques for Product Isolation

Solid Separation--

The first step in product isolation is the separation of the solid and liquid components of the fermentation broth. For intracellular products the cells may be lysed before or after the separation. The current techniques in common use are:

- Sedimentation/Flotation - Filamentous or mat forming organisms can be easily settled and the supernatant broth decanted. In some cases, the cells will float to the top and can be skimmed off the surface. Both of these methods are commonly used in the alcoholic beverage industry and in wastewater treatment.

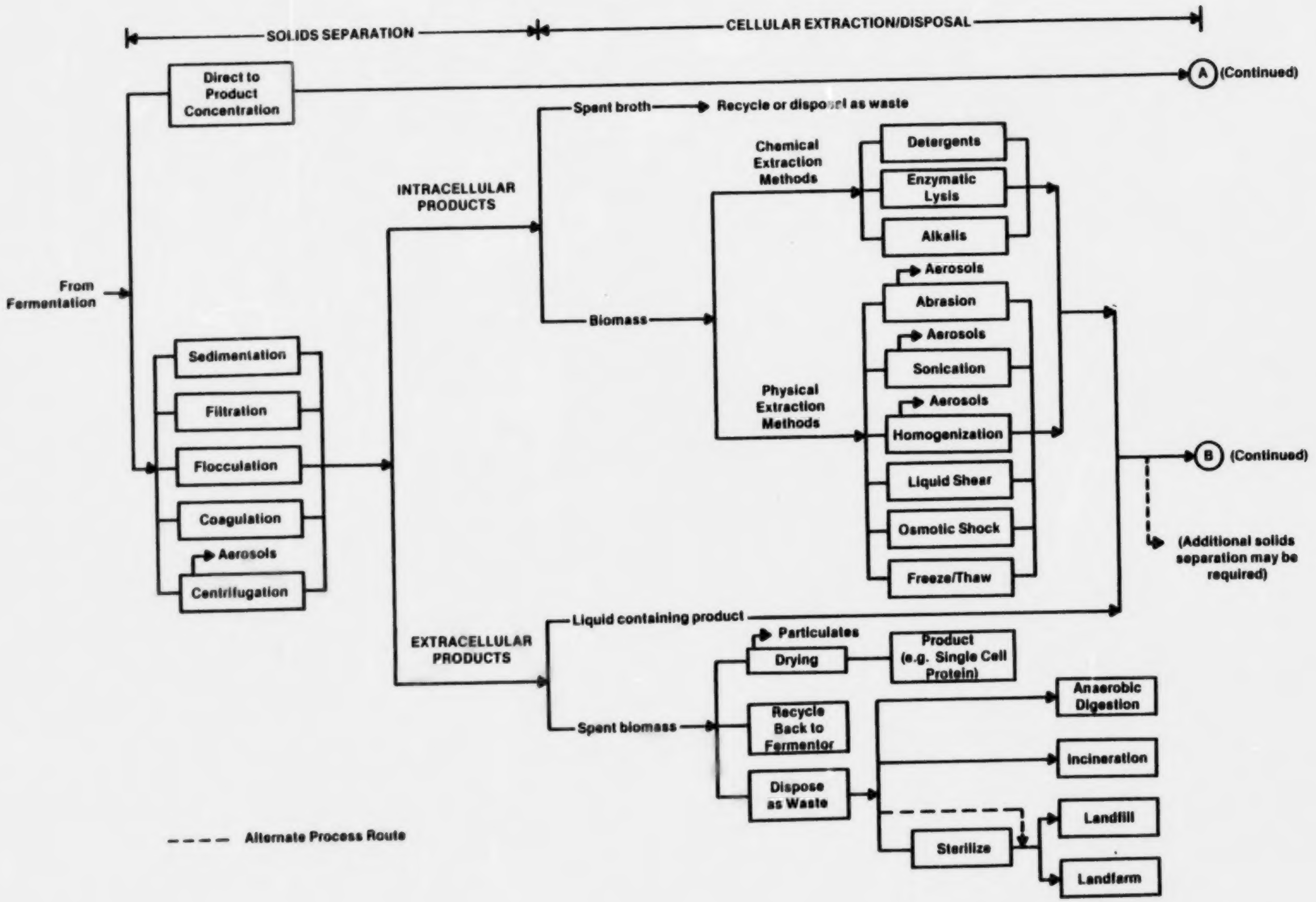

SOLIDS SEPARATION
CELLULAR EXTRACTION/DISPOSAL
From Fermentation
Direct to Product Concentration
A (Continued)
Sedimentation
Filtration
Flocculation
Coagulation
Aerosols
Centrifugation
INTRACELLULAR PRODUCTS
EXTRACELLULAR PRODUCTS
Spent broth
Recycle or disposal as waste
Biomass
Chemical Extraction Methods
Detergents
Enzymatic Lysis
Alkalis
Physical Extraction Methods
Aerosols
Abrasion
Aerosols
Sonication
Aerosols
Homogenization
Liquid Shear
Osmotic Shock
Freeze/Thaw
B (Continued)
(Additional solids separation may be required)
Liquid containing product
Spent biomass
Particulates
Drying
Product (e.g. Single Cell Protein)
Recycle Back to Fermentor
Dispose as Waste
Anaerobic Digestion
Incineration
Sterilize
Landfill
Landfarm
Alternate Process Route

Figure 8. Product Recovery and Purification Processes. (Continued next page.)

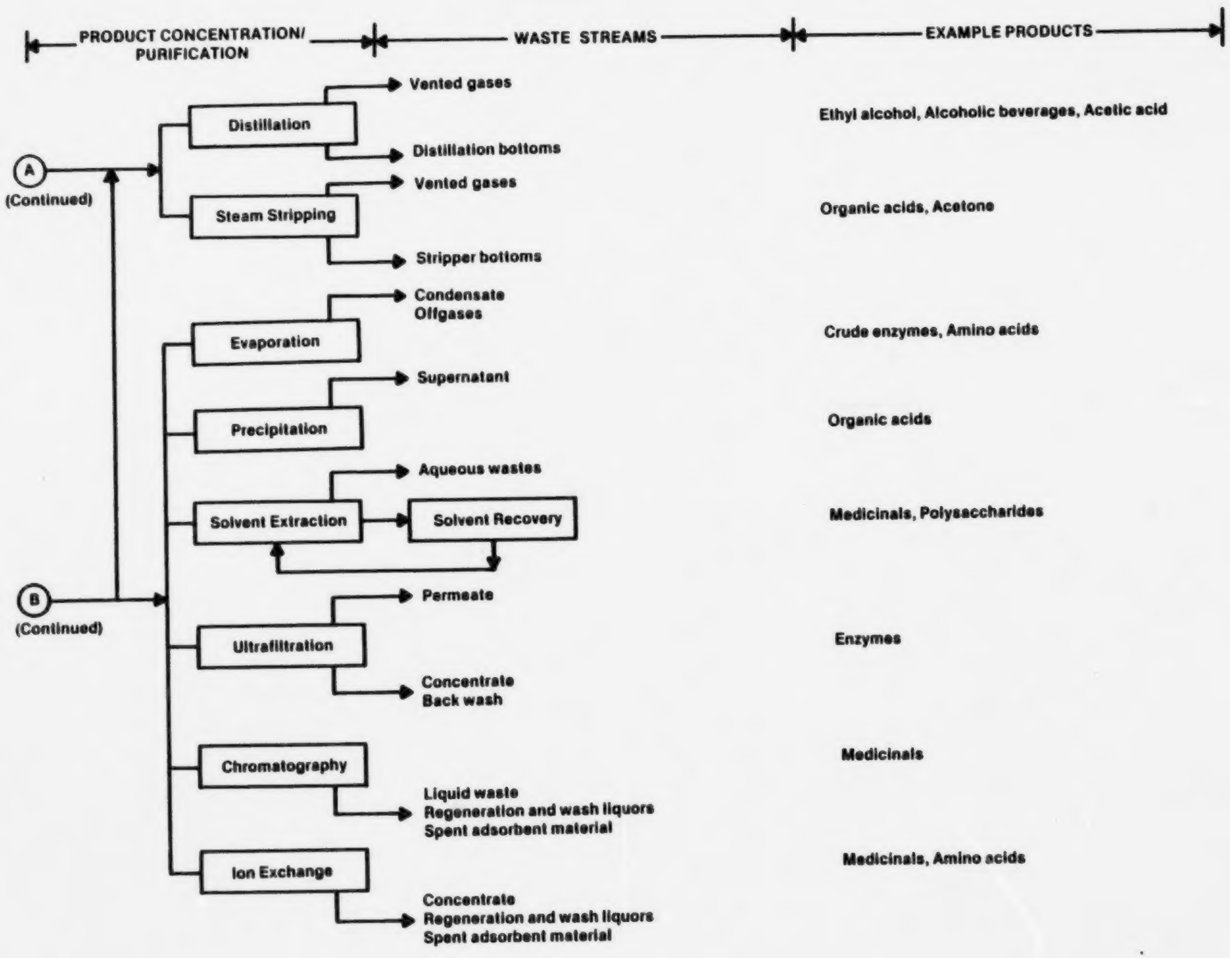

PRODUCT CONCENTRATION/ PURIFICATION
WASTE STREAMS
EXAMPLE PRODUCTS
A
(Continued)
B
(Continued)
Distillation
Vented gases
Distillation bottoms
Ethyl alcohol, Alcoholic beverages, Acetic acid
Steam Stripping
Vented gases
Stripper bottoms
Organic acids, Acetone
Evaporation
Condensate
Offgases
Crude enzymes, Amino acids
Precipitation
Supernatant
Organic acids
Solvent Extraction
Aqueous wastes
Solvent Recovery
Medicinals, Polysaccharides
Ultrafiltration
Permeate
Concentrate
Back wash
Enzymes
Chromatography
Liquid waste
Regeneration and wash liquors
Spent adsorbent material
Medicinals
Ion Exchange
Concentrate
Regeneration and wash liquors
Spent adsorbent material
Medicinals, Amino acids

Figure 8. (Continued)

- Filtration - The most common forms of industrial filters are the plate and frame press and the rotary drum filters. The plate and frame press consists of filter cloths enclosed between corrugated plates. Generally this is applied to small volume fermenters with relatively low solids (1-5 percent). The rotary drum filters are the workhorses of the fermentation industry and are used for large volume filtration with higher solids. The filter media used is dependent on the characteristics of the broth and the degree of clarity of the product. Precoating of the filter may be necessary for less easily filtered broth.

- Flocculation - A flocculating agent may be added to the fermentation broth to help aggregation of fine particles to aid in subsequent filtration or centrifugation. Many natural and synthetic polymers such as gelatine and polyacrylamides and many inorganic salts such as alum, iron, and calcium salts are effective flocculants of microbial cells.

- Coagulation - Inorganic and organic polyelectrolytes may be used as coagulation aids to neutralize the charge on the cells and cause direct adhesion of the particles. Alum is effective as a coagulant for carbonaceous components and iron sulfate or iron chloride are effective for proteins.[19]

- Centrifugation - Centrifugation is a common practice on the laboratory scale because the equipment is readily available. Satisfactory large-scale centrifugation is dependent on the properties of the phases to be separated. Adequate equipment is available for separation of the heavier solids in a relatively light broth. But in actual practice, the biological materials are often small and of low density while the fermentation broths are often viscous and of high density. Satisfactory separation of these materials would require ultra high speed centrifugation which is not available on a large scale. The more commonly used commercial-scale centrifuges include tubular bowl, multichamber disk, solid bowl scroll, and perforated bowl basket centrifuges. Ideally, industrial centrifuges need to be of the continuous flow-through variety.

Cellular Extraction--

Cell disruption is required to release intracellular products and in some cases is also desired as a broth pretreatment step to improve handling characteristics prior to filtration or centrifugation. Cell disruption may be achieved by chemical or physical methods. The choice of method is dependent on the sensitivity of the product. Mechanical methods which generate excess heat may result in the denaturation or inactivation of the product, especially enzymes. Chemical methods need to be compatible with the product and ideally, should leave a flocculated solid phase and a recyclable broth (chemical agents which are heat labile would be destroyed in the recycle sterilization). For production operations a rapid action is desired to avoid long holding times. Currently glucose isomerase and single cell protein (S.C.P.) are the only large-scale productions of intracellular products. The disrupted cell mixture may be passed directly to product concentration/purification; however, additional solids separation may be required.

Chemical Extractions--

- Detergents - Anionic, cationic, and nonionic detergents are used for enzyme extraction. The detergents combine with and solubilize the lipoproteins of cell membranes. Detergents are less than ideal extracting agents for some enzymes and can result in their inactivation by dewatering or precipitating the protein.
- Enzymatic Lysis - Enzymes are very gentle and selective, but expensive, methods of disrupting bacterial and fungal cells. Lysozyme is the most widely known lytic enzyme and is used in the commercial extraction of glucose isomerase from Streptomyces. Lysozyme causes hydrolysis of the polysaccharides in the cell walls of bacteria and is commercially produced from egg white.
- Alkali - Short exposure at pH 11-12.5 results in hydrolysis of cell walls of some bacteria. Application of the method is dependent on the stability of the product at elevated pH levels.

Physical Extractions--

- Abrasion - Ball mills and rapidly vibrating vessels containing glass beads provide grinding and shear forces because of velocity gradients between the organisms and the beads or balls. The rate of stirring or vibration, the ratio of beads to cells, the size of beads, and the contact time influence the extent of cellular fracture. The principal disadvantage is the overheating of the product which can be avoided by working in a cold room.

- Sonication - Ultrasonic treatment at frequencies above 20 kHz results in gaseous cavitation and formation of shock waves which break open the cells of many types of bacteria. Treatment success is dependent on pH, temperature, and ionic strength of the broth and is better suited for use at laboratory scale than large scale.

- Homogenization - Homogenization is a relatively violent means of cell disruption used on the tissues of higher plants and animals and on multicellular algae. On a small scale Waring blenders are commonly used; on large scale modified hammer mills are used.

- Liquid Shear - When suspensions are forced at high pressure through a small orifice, the rapid pressure drop causes the cells to disrupt. The Mantor-Gaulin homogenizer uses a positive displacement pump to pass liquid suspensions through an adjustable valve at pressures up to 8000 psi and out through an orifice. Usual practice is to cool cell suspensions to prevent overheating during processing.[12]

- Osmotic Shock - Osmotic shock caused by suspended cells in buffered solutions of high osmotic pressure (such as 20 percent sucrose) will cause cell bursting in the more delicate Gram-negative bacteria.

- Freeze-Thaw - The formation and melting of ice crystals during freezing and thawing of cell pastes causes sufficient cell disruption to release some proteins.

4.3.2 Product Concentration and Purification

In general, product concentration and purification are the most costly processes in the manufacture of a product and represent about 40 percent of the "added value" (i.e., total cost less cost of purchased materials).[4] Consequently, the value of the product dictates the extent of purification and the choice of techniques. In most cases, the product is purified as little as necessary to meet the demands of the customer or regulatory authority.

Techniques commonly used in pharmaceutical production include solvent extraction, direct precipitation, and ion exchange.[2] These techniques plus five others are described in this section. One or more of the techniques plus additional solids separation steps may be used for a particular product. Some methods may be used as a batch process, while others can also be used on a continuous or semi-continuous basis.

- Distillation - Volatile organic compounds such as alcohols, acetone, and acetic acid are concentrated and purified by distillation. Initial distillation may be in the presence of broth solids, and redistillation may be necessary to separate out components and to further concentrate the product.

- Steam Stripping - Alternatively to the initial distillation, volatile alcohols and acetone may be steam stripped for separation from the broth solids. Separation of components and product concentration is achieved by subsequent distillation. The steam stripping process may also be used to recover solvents used for solvent extraction of the product.

- Evaporation - Crude enzymes and amino acids are concentrated by evaporation in vessels that are often similar to stills or reboilers except that vapor components are not necessarily recovered or recycled except in cases of high value.

- Precipitation - Organic acids are commonly precipitated with calcium salts, and enzymes are "salted out" using ammonium sulfate. Final product removal may also require filtration or centrifugation.

- Solvent Extraction - Solvent extraction by liquid-liquid partition is a common method used for concentration and purification of antibiotics. Sequential extraction may be necessary to remove contaminants. Solvent separation from the product can be done either by precipitation, solvent evaporation, or steam stripping. Normally solvents are recovered and reused. Typical solvents used in the pharmaceutical industry are benzene, chloroform, 1,1-dichloroethylene, and 1,2-transdichloroethylene.[2]

- Ultrafiltration - Ultrafiltration hydraulically separates high molecular weight components from low molecular components through membranes of very small pore size. Typical configurations of membranes include hollow fiber or tubular bore cylinders, spiral wound, or plate and frame flat sheets. Large molecules such as yeasts, bacteria, and viruses with dimensions ranging from 30 to 10,000 nm in diameter can be separated and recycled while the smaller proteins, enzymes, antibiotics, and organic acids with dimensions less than 10 nm pass through the membrane as the permeate.[20] Ideally, the smaller molecules can then be separated by subsequent ultrafiltration using membranes with decreasing pore sizes.

- Chromatography - Purification by chromatography is based on the general principle of binding solute molecules on a solid substrate as a solution is passed through a column and the subsequent elution of the solute. Materials used for the solid substrate determine the porosity of the column and the solute molecules retained. Cross-linked dextrans, agarose, and polyacrylamides are frequently used for enzyme purification. Specific affinity adsorptive methods using monoclonal antibodies and DNA probes are currently in the experimental stages and have potential for commercial development for purification of products of high value.

- Ion Exchange - Traditional anionic and cationic ion-exchange resins with sulfate, carbonate, or ammonia functional groups have high capacity for protein adsorption and thus can be used for product concentration and purification. Usage is limited to products which are not sensitive to the extremes in pH or ionic strength of the solutions used for the subsequent elution process and for the removal of inorganic contaminants.

4.3.3 Waste Streams from Product Recovery and Purification

Waste streams associated with product recovery and purification from fermentation processes are summarized in Table 8. The liquid wastes are typically characterized as being high in BOD, COD, and TSS with a pH range between 4.0 and 8.0.[2]

Spent broth wastes contain substantial concentrations of unused organic substrates such as sugars and starches as well as residual nitrogen and phosphates added initially as nutrients to the fermentation broth. The characteristics and concentration of the residual organics are dependent on the original substrate, the type of product, and the conditions of the fermentation. Fermentation wastes (spent broth and/or spent biomass) will also contain viable microorganisms, any pesticide or metal residues which may have entered the system with the feedstock, product residues, product intermediates, and any solvents or metals added during the process of product recovery and purification. Gaseous emissions containing hydrocarbons may also be generated from product recovery and purification processes such as distillation, steam stripping, and evaporation.

TABLE 8. WASTE STREAMS ASSOCIATED WITH PRODUCT RECOVERY AND PURIFICATION

Unit	Waste Stream	Potential Contaminants	Current Treatment/ Disposal Methods
SOLIDS SEPARATION			
General	spent broth	unused organic substrate substrate contaminants (e.g., pesticides) unused nutrients residual product product intermediates viable microorganisms color	conventional biological wastewater treatment with pretreatment for specific pollutant removal
	spent biomass	concentrated viable microorganisms spent broth contaminants	recovered as by-product sterilization anaerobic digestion landfarming landfill incineration
Centrifugation	aerosols	viable microorganisms/ enzymes volatile organics	containment/treatment of air emissions by filtration/sterilization
CELLULAR EXTRACTION			
Abrasion, Sonication, Homogenization	aerosols	viable microorganisms/ enzymes volatile organics	containment/treatment of air emissions by filtration/sterilization
PRODUCT CONCENTRATION/ PURIFICATION			
Distillation	distillation bottoms	unused organic substrate substrate contaminants (e.g., pesticides) unused nutrients residual product product intermediates viable microorganisms/ enzymes cellular debris (proteins, lipids) color high BOD, COD, TSS	conventional biological wastewater treatment with pretreatment for specific pollutant removal
	vented gases	volatile organics	vented to atmosphere incinerated flared utilized as air feed to boilers
Steam Stripping	stripper bottoms	Same as distillation bottoms	conventional biological wastewater treatment with pretreatment for specific pollutant removal
	vented gases	volatile organics	vented to atmosphere incinerated flared utilized with air feed to boilers

(Continued)

TABLE 8. (Continued)

Unit	Waste Stream	Potential Contaminants	Current Treatment/ Disposal Methods
Evaporation	condensate	volatile organics and inorganics	steam stripping followed by conventional biological wastewater treatment
	offgases	volatile organics	incinerated flared utilized with air feed to boilers
Precipitation	supernatant	Same as distillation bottoms plus residual precipitating agents (Fe, Al, Cu, Zn, Ca) Reagents used for cellular extraction (detergents, enzymes, alkalies)	conventional biological wastewater treatment may require pretreatment to adjust pH or precipitate residual metals
Solvent Extraction	aqueous phase waste stream	Same as distillation bottoms plus residual solvent and reagents used in cellular extraction (detergents, enzymes, alkalies)	steam stripping to remove residual solvent conventional biological wastewater treatment
Ultrafiltration	permeate	Same as distillation bottoms, low solids	conventional biological wastewater treatment with pretreatment for specific pollutant removal
	concentrate	Same as distillation bottoms, potentially high solids	conventional biological wastewater treatment with pretreatment for specific pollutant removal
	backwash	Same as distillation bottoms, high solids	conventional biological wastewater treatment with pretreatement for specific pollutant removal
	spent filter	filter media, high solids, viable microorganisms	incineration
Chromatography	liquid waste	Same as distillation bottoms	conventional biological wastewater treatment with pretreatment for specific pollutant removal
	regeneration and wash liquors	solvents, acids, bases, residual product	no data
	spent adsorbent material	adsorbent product residue	no data

(Continued)

TABLE 8. (Continued)

Unit	Waste Stream	Potential Contaminants	Current Treatment/ Disposal Methods
Ion Exchange	concentrate	Same as distillation bottoms	conventional biological wastewater treatment with pretreatment for specific pollutant removal
	regeneration and wash liquors	acids, bases, residual product	no data
	spent adsorbent material	resins	no data

Spent broth and spent biomass may be recycled back to the fermenter. However, solvents, detergents, metal precipitation agents, and other additives may make the waste unfit for recycle and require disposal. In general, spent broths are treated by centralized conventional biological treatment with pretreatment of isolated waste streams for specific pollutant removal as necessary.[2] Spent biomass can be treated by anaerobic digestion to reduce solids, with the remaining solids used as feed or disposed of by landfill, landfarming, or incineration. However, incineration can be expensive due to the high moisture content of biomass filter cakes and solids. The effectiveness of these treatment methods is discussed in Section 6.0, "Potential Impacts and Control Technologies Associated with Biotechnology Waste Streams."

Special problems may be encountered because these streams can contain high concentrations of viable microorganisms. Even in cases where the microorganism is of relatively low pathogenicity (many microbiologists consider all bacteria potentially pathogenic as opportunists), the release of very large numbers of viable organisms may pose a threat to the environmental balance of a localized area and create conditions which are unpleasant or generate quantities of undesirable materials (e.g., antibiotics/as growth of the microorganisms continue).[21]

Problems may be compounded with the release of microorganisms of higher pathogenicity and may be a threat to worker health and safety within the plant and the community-at-large in the event there is any breakdown in normal environmental sanitation controls.

The use of genetically altered organisms could represent a more serious threat as the risks associated with their release into the environment and methods to measure these risks are largely unknown. Tests and models are available for the prediction of risks associated with the release of toxic chemicals, but it is not known if these same methods can also be used for assessing microorganisms and their synergistic and antagonistic relationships with the indigenous population.

SECTION 5

UNCONTAINED AND SEMI-CONTAINED BIOTECHNOLOGY PROCESSES

In contrast with the controlled processes described in the previous section, uncontained and semi-contained processes involve use of microorganisms outside of a contained structure or to effect changes other than fermentaton conversions. Generally, these processes fall into the following categories:

- Microbiological Metal Extraction and Metal Accumulation/Concentration,
- Energy Production, and
- Nontraditional Pollution Control.

These processes may present special environmental concerns since their waste products either are not well defined or primarily are uncontrolled.

The following sections provide an overview of uncontained processes that now appear to be commercially viable, along with a description of their waste streams and associated control technologies. In many instances, adequate data on potential waste streams and control technologies do not exist.

5.1 MICROBIOLOGICAL METAL EXTRACTION AND METAL ACCUMULATION/CONCENTRATION

Microorganisms are used for two principal purposes by the mining industry: to extract metals from metal ore and to recover metal ions from solutions such as wastewaters. The second purpose can be considered both as metals recovery and pollution control. The following sections describe the processes associated with each of these uses.

5.1.1 Metal Extraction

Extraction of metals from ore, or bacterial leaching as it is called, has been practiced for centuries, primarily to obtain copper from copper sulfide ore. Microbiologically-extracted copper now accounts for 10-12 percent of the copper produced in the United States.[22] Recently, bacterial leaching has been used with uranium and other ores as well.

Bacterial leaching can take place in an ore dump (open pile), on an ore heap, or in-situ in a mine. In some cases, the dump leaching takes place in a tank or vat. Of the three types, dump leaching in which a leaching solution is sprayed on an open dump is the most uncontrolled and, therefore, would have the greatest impact on the environment.[22] Heap leaching usually takes place in a structure built on an impermeable liner. In-situ leaching involves the direct application of acidified water to a very limited area on the walls and ceilings of a mine.

In general, biological extraction processes are less energy intensive and less polluting with respect to conventional pollutants than are traditional methods. They also are better suited to removing metals from low grade ores (for example, ores of tailing with less than 0.5 percent copper) than are the traditional methods. On the other hand, biological extraction processes generally are slower and less efficient at extracting metals than traditional processes--75 percent recovery versus 95 percent for traditional processes.[23] It is also difficult to predict the yield from microbiological extraction.

Microbiological metals extraction can be broken down into two types: direct and indirect. Direct microbiological leaching involves bacterial oxidation of the metallic portions of the ore. This oxidation solubilizes the metal and allows it to be washed out of the ore. With indirect, or bacterially assisted leaching, the bacteria oxidize ferrous iron in metal ore; the resulting ferric iron is itself an oxidizing agent that solubilizes other metals in a sulfuric acid solution.

Figure 9 shows the steps involved in biological metal extraction processes. With both types of extraction, direct and indirect, acidified water is sprayed on the dump (or on mine walls, in the case of in-situ extraction). The water percolating through the dump causes the bacteria to multiply; the bacteria utilize the carbon dioxide from the air in the acid water spray and the energy released by oxidation of inorganic substances to increase in number. The oxidized metals are washed out of the dump with the leach water and into catch basins. From there, the leach water goes into an electrolysis, precipitation, or cementation process where the metal (e.g., copper or uranium) is recovered. In the cementation reaction, ferrous iron replaces the copper in the solution. In some cases, solvent extraction precedes the precipitation process. After removal of the metal, the leach water is recycled to the dump.

The bacteria most often used for both direct and indirect metals leaching belong to the genus *Thiobacillus*. They are indigenous to sulfide ores and are considered to be chemolithotrophic ("rock eating") because they produce energy by oxidizing inorganic materials. Although the *Thiobacillus ferrooxidans* species is the most widely used for bacterial leaching, *T. thiooxidans*, a combination of *T. ferrooxidans* and *T. thiooxidans*, *Sulfolobus*, a combination of *Leptospirillium ferrooxidans*, and TH (thermophilic and Thiobacillus-like) microorganisms also are used.[22]

T. ferrooxidans are acidophilic (i.e., acid-loving) and thrive in sulfide ores with a high concentration of sulfuric acid. In fact, all of the metal leaching bacteria thrive under conditions that are inhospitable to most microorganisms: high concentrations of sulfuric acid and soluble metals. Highly acidic conditions must be maintained in the leach dump or heap to favor leaching activity, which is dependent on the amount of ferric iron and other metals in solution. By contrast, high concentrations of certain metal ions, such as silver and mercury, lack of air, and extremely high temperatures can be fatal to the microorganisms.

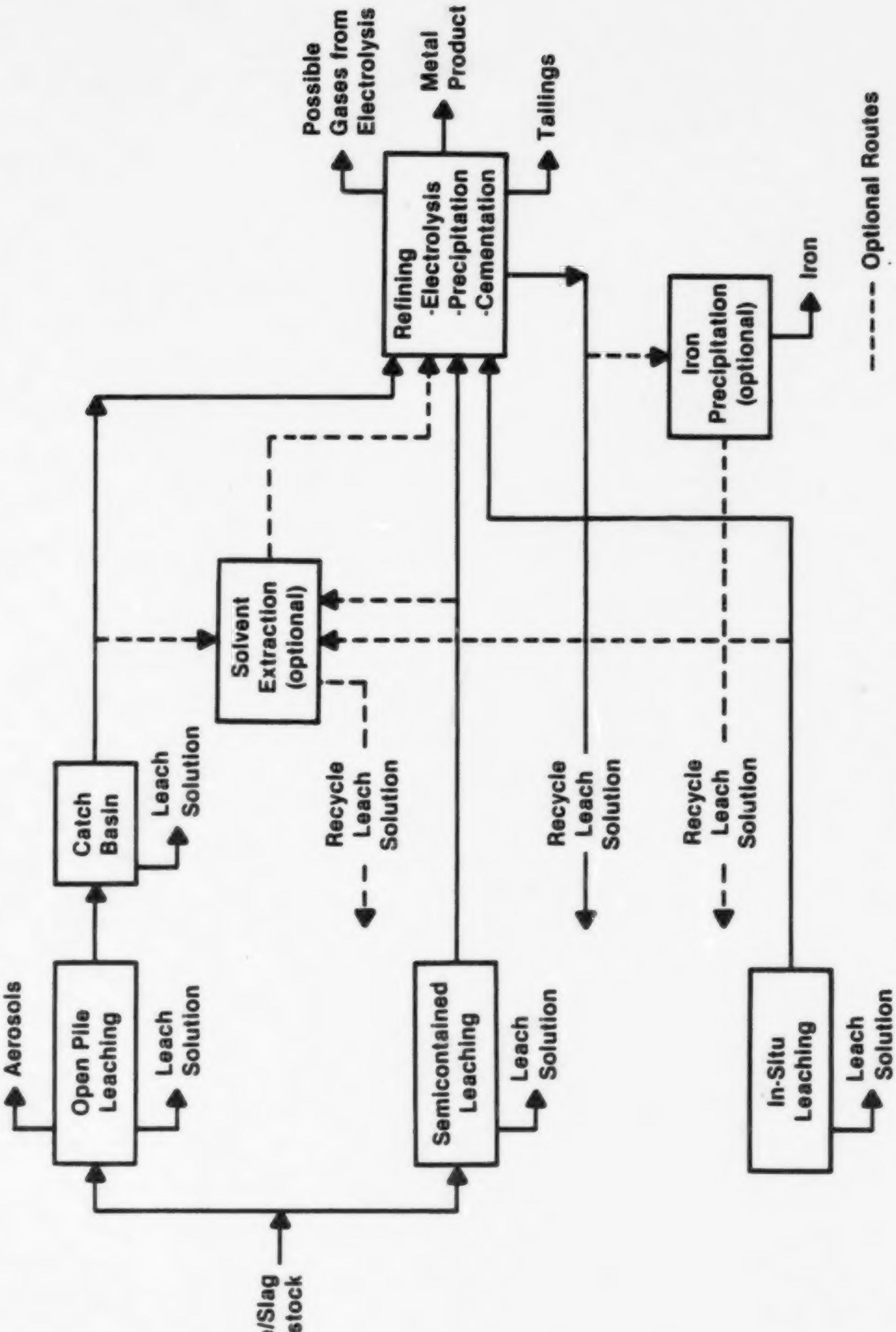

Ore/Slag Feedstock
Open Pile Leaching
Aerosols
Leach Solution
Catch Basin
Leach Solution
Semicontained Leaching
Leach Solution
In-Situ Leaching
Leach Solution
Solvent Extraction (optional)
Recycle Leach Solution
Recycle Leach Solution
Recycle Leach Solution
Refining
-Electrolysis
-Precipitation
-Cementation
Possible Gases from Electrolysis
Metal Product
Tailings
Iron Precipitation (optional)
Iron
------ Optional Routes

Figure 9. Biological Metal Extraction Process.

To date, very little, if any, genetic manipulation of the bacteria used for metals extraction has taken place. Since only indigenous bacteria are used in the extraction process, there has been no selection or transfer of bacteria from other sources. However, researchers have identified several areas of bacterial improvement that would enhance extraction processes. These include increased tolerance of heat, acid, toxic metals, and lack of oxygen and increased ability to leach metals.

In a typical large commercial microbiological dumping operation, millions and sometimes billions of tons of ore are transported from the mine to the dump site. In the past, dump sites have been poorly planned and crudely constructed, making their operation somewhat uncontrolled. Many are built on the side of a hill or in a valley to make the ore pile stable and to facilitate recovery of metals from the bottom of the pile. However, many are located on permeable ground which means that leach water spills cannot be contained. Others are conical in shape, which limits surface area and thus the amount of air that can reach the accumulated ore.

Dump leaching processes could be improved by making both biological and structural changes in the way the processes now operate. Almost all leaching bacteria are aerobic. This creates a problem in very large dump sites where virtually no oxygen is able to penetrate to the center of the dump. Solutions to this problem include genetic manipulation of bacteria to develop an anaerobic metal leaching strain or using aeration or fingering systems to introduce additional air into the dump.[23] The design of a dump also could be altered to allow maximum surface area or to make leachate collection easier.

Heap leaching takes place in concave structures especially designed for the process, rather than the ore and waste tailing piles in which dump leaching occurs. Heaps are constructed above impermeable liners, which greatly reduces the possibility of a spill of the leach solution. In general, the ore placed in the heap leaching structure is of a higher quality than that found in the dump and is crushed prior to leaching. Sometimes aeration systems are used to inject additional air into the interior of the heap, thereby enhancing the bacteria's ability to leach the metals.

Several variations of in-situ bacterial leaching exist. Leach solution may be sprayed on the surface above an underground mine and allowed to percolate down into the mine chambers where it is collected. Leach solution also may be applied directly to mine walls and ceilings or to rubble in the mine. In-situ microbiological mining appears to be very well suited to recovery of metals from low grade ores in relatively inaccessible locations.

Table 9 lists the extraction processes and associated microorganisms available for leaching eleven metals. Note that many of these have only been tested in the laboratory. Currently only copper and uranium bacterial leaching processes are in commercial use. Microbiological extraction processes also appear to be promising for recovery of precious and platinum group metals, manganese, nickel, cobalt, and other metals that can be found in large quantities of low grade ore and ore wastes. This promise, however, is highly dependent on present and predicted market conditions, including the availability and price of the metals.

5.1.2 Accumulation/Concentration of Metal Ions

Closely related to their ability to leach metals from ore and other solid waste is the capacity of certain bacteria to accumulate inorganic ions from seawater and wastewaters. Some types of fungi and algae have a similar capacity. This accumulation takes three forms:

- adsorption of metal ions on the surface of the microorganisms,
- uptake of metals within the cells of the microorganisms, and
- chemical transformation of the metals.

Technologies that utilize these abilities generally are still in the developmental stages. Where they do exist, they are often included as part of

TABLE 9. MICROBIOLOGICAL METALS EXTRACTION MECHANISM

Extraction	Microorganism
COPPER LEACHING	
Dump/Heap	
Japanese patent	iron-sensitive bacteria
Chalcopyrite	T. ferrooxidans, T. thiooxidans
Leaching of copper and nickel using acid iron solution	T. ferrooxidans and Beijerinckia lacticogenes
High Temperature Leaching	Ferrolobus
Tank	
Biological Acid Leaching using silver as a catalyst	T. ferrooxidans
In-Situ	
Water sprayed on ground surface; underground mine passages used as drainage chambers	T. ferrooxidans
URANIUM LEACHING	
In-Situ	
Indirect process to remove uranium from brannerite	No data
*Capillary leaching	
Heap/Columns	
Ferric sulfate	T. ferrooxidans
MANGANESE	
Dump	
*Bacterial leaching	T. thiooxidans
*Leach/falling air-exposure	no data

(Continued)

TABLE 9. MICROBIOLOGICAL METALS EXTRACTION PROCESSES (Continued)

Extraction	Microorganism
GOLD	
Heap	
*Cyanide	no data
LEAD AND ZINC	
Dump	
Bacteria leaching	T. ferrooxidans
*Leaching of zinc from sphalerite and sulfide concentrations	combination of T. ferrooxidans and T. thiooxidans
NICKEL AND COBALT	
*Bacterial leaching	T. thiooxidans
MOLYBDENUM AND TUNGSTEN	
*Leaching of molybdenum from molybdenite concentrate	unidentified thermo-tolerant bacterium
ALUMINUM	
*Leaching from bauxite medium	Bacillus circulans
TIN	
Vat leaching process of residues left after gold leaching of arsenopyrite	no data

*Laboratory testing only.

Reference: 1, 24.

established pollution control technologies: however for this report, accumulation of metals is categorized as a mining process. The various metals accumulation processes are illustrated in Figure 10.

The basic mechanism that allows microorganisms to adsorb metal ions is the negative charge of their cell membranes and cell walls which attract positively charged metal ions in wastewater or other solutions. Other ill defined cell surface mechanisms also seem to help microorganisms adsorb metals. Table 10 lists microorganisms that have been identified as adsorbing specific metal ions. One of these microorganisms, R. arrhizus, has been shown to adsorb uranium at more than twice the rate of a commercially used ion-exchange resin.[22]

Microorganisms take up metal ions intracellularly because they require the ions in order to perform some cellular activity. In some cases, they absorb such high concentrations of toxic metals that they die. Pseudomonas aerigunosa exhibits this kind of behavior with uranium.[22]

Chemical transformation processes carried out by microorganisms include precipitation of metals into chelates and incorporation of metals into volatile compounds that eventually evaporate. Its precipitation ability may enable a microorganism such as T. ferrooxidans to be used to pretreat acid mine drainage water prior to neutralization.

Microbiological metals accumulation has been demonstrated both in laboratory studies and in natural settings, such as metal tailings and waste treatment ponds. In such ponds, strains of algae such as Spirogyra, Rhizoclonium, Hydrodictyon, Cladophora, Chlorella pyrenoidosa, and Scenedesmus quadricauda accumulate soluble ions of metals such as molybdenum, selenium, uranium, and radium.[22,25] Microorganisms then cause the algae to decay, producing hydrogen sulfide which in turn causes metals to precipitate as sulfides. Pure cultures of blue and green algae have been used in conjunction with traditional waste treatment technologies to remove arsenic, cadmium, zinc, mercury, and lead from wastewaters.

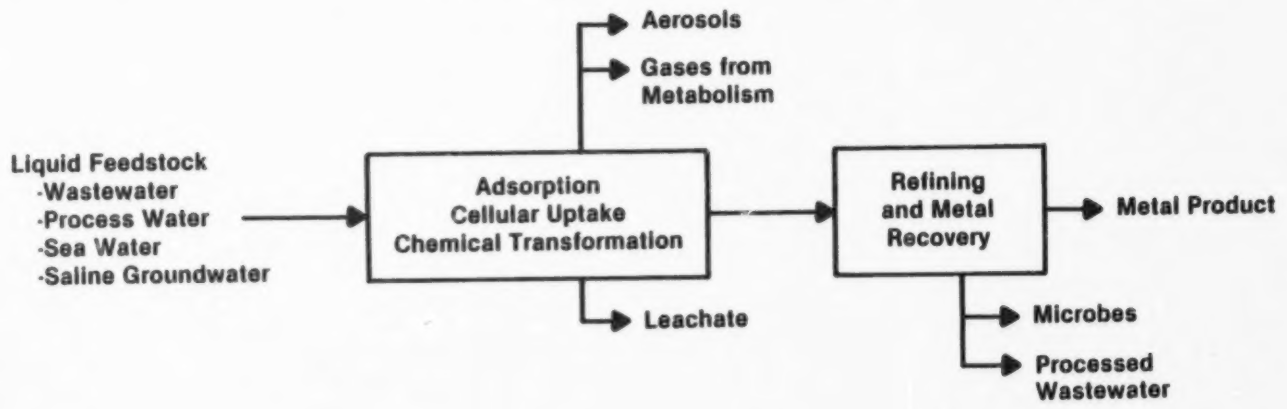

Aerosols
Gases from Metabolism
Liquid Feedstock
-Wastewater
-Process Water
-Sea Water
-Saline Groundwater
Adsorption
Cellular Uptake
Chemical Transformation
Leachate
Refining and Metal Recovery
Metal Product
Microbes
Processed Wastewater

Figure 10. Metals Accumulation Processes.

TABLE 10. SOME METAL ADSORBING MICROORGANISMS

Microorganisms	Metal Adsorbed
Saccharomyces cerevisiae	Uranium
Rhizopus arrhizus	Uranium
Escherichia coli	Variety of metals
Bacillus subtilis	Variety of metals
Sphaerotilus Leptothrix	Manganese, iron
Hyphomicrobium	Manganese
Gallionella	Iron

These metal accumulation mechanisms show promise for commercial wastewater treatment and metal recovery purposes. Like metals extraction procedures, these microbiological functions could benefit from a carefully controlled setting (e.g., pond or other structure design) and genetic modifications to increase the specificity of microorganisms for accumulating particular metals.

5.1.3 Waste Streams Associated with Microbial Mining

Although much has been written about the development and use of microbiological mining techniques, very little specific information is available regarding potential waste streams. Many of these processes, such as leaching of copper ore and metal uptake by algae have been taking place in a natural setting for years, and, in some cases, centuries. Despite this fact, the precise workings of the biological and biochemical reactions involved remain uncertain.

Taking into consideration some of these data gaps, some potential waste streams associated with various microbiological mining processes have been identified. These are presented in Table 11.

In the case of metals extraction processes, the primary waste streams of concern are associated with dump leaching. Aerosols and other emissions are generated by the spraying of the ore dumps and the surface of the dump itself. Acidic leach solution could become a harmful effluent should it spill before or during recycle.

Aerosols are produced by the acidic water that is sprayed or sprinkled on open dumps. Since this spray is made up of recycled leach solution, it contains the leaching microorganisms in a dilute sulfuric acid solution. Rain falling on the dump also will produce aerosols and some aerosols and emissions are caused by frictional turbulence and thermal currents.[26]

TABLE 11. WASTE STREAMS/CONTROLS OF MICROBIOLOGICAL MINING PROCESSES

Process	Waste Stream	Potential Contaminants	Current Treatment/ Disposal Method
METALS EXTRACTION			
Open Pile	Aerosols from acidic water spray	H_2SO_4 microorganisms	containment/ filtration
	Leach solution	microorganisms H_2SO_4 organic acids	recycle use of impermeable liners for pile site pH adjustment
	Ore tailings	metals trace elements	land disposal
Semi-Contained Leach Tank	Leach solution	microorganisms H_2SO_4	recycle
In-Situ	Leach solution	microorganisms H_2SO_4 organic acids	recycle
Refining	metal tailings electrolysis off gases	trace metals inorganic gases (e.g.,Cl_2)	landfill scrubbing
Iron Precipitation	iron	iron trace metals	landfill
METALS ACCUMULATION			
Adsorption	aerosols and possibly gases from metabolism	microorganisms trace elements	no data
	pond leachate	microorganisms metals trace elements	containment/ filtration
Intracellular Uptake	aerosols and possibly gases from metabolism	microorganisms	no data
	toxic heavy metals accumulated in microorganisms/algae	heavy metals microorganisms/ algae	land disposal
	pond leachate	metals trace elements microorganisms	containment/ filtration
Chemical and Biotransformation	aerosols/emissions	microorganisms trace elements	no data
	pond leachate	metals trace elements microorganisms	containment/ filtration

References: 22, 23.

Aerosols from leaching dumps potentially could be transported long distances. This introduces the problem of how the microorganisms and dilute sulfuric acid might affect humans and plant life near the dump and at some distance away from it. In general, the ability of a microorganism to survive in an aerosol and to be able to eventually infect a host depends on four factors: the type of organism; conditions which generate the aerosol; the duration of and conditions within the aerosol; and the final growth medium.[26]

Harmful effects associated with any uncontrolled release of microorganisms include the potential of some to cause disease in plants, animals, and humans, and the possibility that all of them could undergo undesirable genotypic or phenotypic changes.[27] Research is needed in order to acquire more knowledge about the mechanisms involved in transport of microorganism-laden aerosols as well as how the microorganisms react with other living organisms.

One-time or ongoing release of microorganism-laden acidic leach solution presents related, but slightly different problems from those associated with aerosols. In surface or groundwater, this leach solution may change the natural balance among various species of flora and fauna. Again, additional research is needed to precisely determine how metal leaching microorganisms affect ground and surface water and soil.

Few data exist regarding potential waste streams of metal accumulation processes since they have never been used on a commercial scale. However, since most of these processes take place in a pond or similar environment, it is expected that aerosols/emissions from the pond surface, leachate from uncontained ponds, and sediment containing high concentrations of toxic metals would be the major waste streams. Source testing is needed to verify the presence and identify contaminants in these waste streams.

The concerns described above regarding aerosols and other discharges from microbiological metals extraction operations also apply to metals accumulation techniques.

Aerosols and other discharges from metals accumulation open ponds may be transported long distances as has been previously noted. Microorganisms and toxic heavy metal-laden waters may be spilled or otherwise leach into the soil and surface/ground water surrounding a pond site. Also, toxic metals and trace elements in pond sediment may leach into the surrounding soil and water as well.

One of the major unknowns in connection with waste streams from metals accumulation operations is how microorganisms affect the soil, water, and air in terms of chemical/biological transformations and accumulation of other potential pollutants. Some study in this area has taken place. At Florida International University, researchers simulated conditions at a local aquifer and revealed the presence of several chloroethene compounds that have never been used in that part of South Florida.[28] These compounds seem to have been formed from other solvents as a result of microbial metabolism. More research is needed to increase understanding of complex biotransformation and chemical transformation processes caused by microorganisms, especially in cases where the transformation product is potentially toxic to humans.

5.1.4 Controls

Since microbiological mining techniques have not been widely used to date (except in the case of copper leaching), there is only a small amount of information available on applicable control technologies. Basically, controls for these processes fall into three categories:

- biological controls,
- physical controls (containment) through engineering design, and
- conventional pollution controls.

Biological control consists of genetic manipulation before the processes begin. Selection and isolation of microorganisms that can survive only within a very narrow range of pH is one example of this type of control. Selection of these microorganisms may involve genetic engineering techniques such as recombinant DNA or gene fusion.

Since most of these processes rely on the recycling of microoganism-laden solutions, it is crucial that the process designs include physical containment provisions. These include catch basins and impermeable liners for metal leaching and metal accumulation operations. Some type of enclosed air filtration system or design provisions to decrease the amount of active surface area in a dump or pond could be used to control aerosols and other discharges. However, the scale of such operations may prohibit the use of such controls.

Worker safety is another major concern, especially considering the high probability that aerosols and other emissions will be inhaled by those working at metal leaching dumps. Before specific controls can be prescribed, however, there is a need for health effects, epidemiological, and toxicological studies.

Many of the metals extraction and accumulation processes take place in open ponds or dumps similar to existing waste treatment ponds and waste disposal sites. Existing methods of pollution control used to reduce air emissions/aerosols and liquid effluents from the latter can be used with microbiological operations as well. However, their effectiveness on such large-scale processes needs to be demonstrated.

5.2 ENERGY PRODUCTION

Microorganisms may be used in a variety of ways either to directly produce fuels or to enhance their production. Most of the direct energy production techniques (ethanol, energy from biomass) are not described in this section because they involve fermentation processes and thus are considered with contained product manufacturing processes. This section describes two groups of processes which are essentially uncontained:

- microbiological enhanced tertiary oil recovery (including recovery from oil shale), and
- direct synthesis of hydrocarbons by microorganisms or higher life forms.

Neither of these process groups has been commercially utilized to any significant degree. Therefore, available information either is based on laboratory and limited field experience or is highly speculative.

5.2.1 Microbiologically Enhanced Oil Recovery

Since an estimated 20 billion barrels of crude oil in the continental U.S. currently remain unrecoverable by primary or secondary means, there is strong motivation for developing tertiary oil recovery methods.[29] Primary oil recovery depends on sufficient pressure in the oil-bearing reservoir to force the oil to the surface. When this pressure drops below a certain level (typically when about 20 percent of the oil in the formation has been removed), primary production ceases. At this point, secondary recovery techniques such as pumping gas into the reservoir or water into the aquifer are used to recover another 10 to 20 percent of the oil. However, when the recovered oil begins to contain increased quantities of the gas or water, these recovery techniques are no longer cost-effective. At this stage, the remaining oil is hard to recover because of its high viscosity, adhesion to rock surfaces, presence in blind cavities, and a low pressure differential in the reservoir.

To solve some of these problems, various thermal, chemical, and microbiological recovery methods have been either used or proposed. Three basic approaches to microbiologically enhanced recovery are as follows:

- injection of nutrients into a reservoir to encourage growth of indigenous microorganisms;
- injection of foreign microorganisms that produce products which increase oil recovery; and
- injection of biotechnology products (e.g., surfactants and biopolymers produced by fermentation processes).

Figure 11 shows the basic steps involved in the first two approaches. Although some researchers feel that the third approach, injection of biotechnology products shows the most promise for tertiary oil recovery, it is not further addressed in this section since the actual application of biotechnology processes occurs during product manufacture (i.e., fermentation). These products (polymers such as xantham gum, biosurfactants, fermentation alcohols, acids, and ketones) are used as chemically enhanced oil recovery agents.

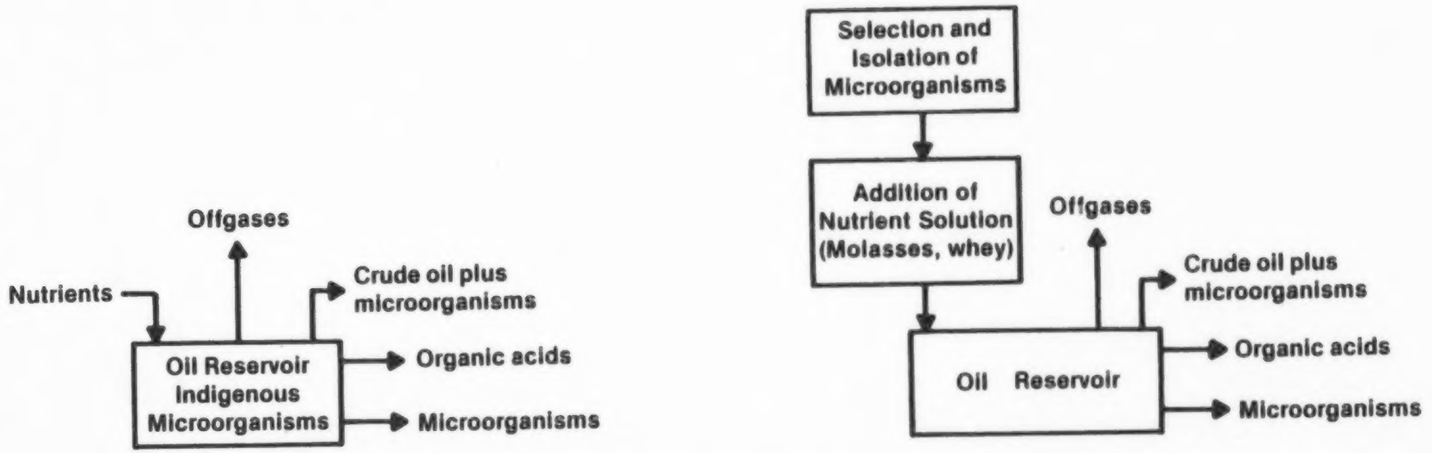
Nutrient Injection to Stimulate In-Situ Microorganisms--

A variety of diverse microorganism populations exist in oil-bearing reservoirs. Many of these have yet to be identified, but both anaerobic and aerobic species have been isolated in core samples. When stimulated by the proper nutrients, these microbes can decrease oil viscosity by producing substances such as polymers, surfactants, or gases.

The nutrients which must be injected to stimulate the anaerobic microorganisms include fermentable substrates such as whey or molasses.[1] Without this nutrient, the microorganism will consume a fraction of the crude oil. In the case of the aerobes, oxygen must be present so that they may metabolize the existing oil. Unfortunately, one drawback to this type of oil recovery is that the microorganisms, once stimulated, may utilize the oil as a substrate and effect a biological conversion which reduces the quality of the oil.

Injection of Microorganisms--

Microorganisms are injected into oil reservoirs with the idea that once in place, they will produce polymers, surfactants, and gases; leach oil from rock matrices; and plug high-permeability zones that prevent repressurization. Additionally, production of gases such as methane, carbon dioxide, and nitrogen can contribute to repressurization of the reservoir and decreased oil viscosity. Organic acids are produced which react with reservoir carbonates to form CO_2 and to modify the structure of the rocks. Surfactants reduce interfacial tension between oil and water in the reservoir.


Nutrients
Offgases
Oil Reservoir Indigenous Microorganisms
Crude oil plus microorganisms
Organic acids
Microorganisms
Selection and Isolation of Microorganisms
Addition of Nutrient Solution (Molasses, whey)
Offgases
Oil Reservoir
Crude oil plus microorganisms
Organic acids
Microorganisms

INJECTION OF NUTRIENTS

INJECTION OF MICROORGANISMS

Figure 11. Microbiologically Enhanced Oil Recovery.

Most of the work on microbiological oil recovery has taken place in eastern Europe--Hungary, Poland, Romania, and Czechoslovakia--and ranges from laboratory studies to field trials. In the United States, the U.S. Department of Energy - Bartlesville Energy Technology Center is sponsoring research through Oklahoma State University.[29]

In the Romanian and Polish field tests, bacterial strains were isolated from produced waters, well-bottomhole mud, soil near oil wells, crude oil, and sugar processing wastes. These bacteria included the following genera: Pseudomonas, Escherichia, Arthrobacter, Mycobacterium, Micrococcus, Peptococcus, and Clostridium. A mixture of two or more of these cultures was injected into oil reservoirs along with a molasses nutrient solution. In the oil fields that responded to this treatment, oil production increased by 16-20 percent. Where production did not increase, it was found to be a result of high salinity, low permeability, or heterogeneity.[29] This has prompted researchers to conclude that injection of a lower salinity water and additional organic nutrients, determination of an optimal fermentation period, and development of analytical procedures for measuring the progress of the in-situ fermentation are necessary for successful use of this type of tertiary oil recovery.

Oil Shale Recovery--

Microorganisms also may be used to enhance the recovery of oil from oil shale. This process, still under development, is more similar to metal leaching than tertiary oil recovery. Microorganisms are used to dissolve the mineral matrix of oil shale and release kerogen.

Most proposed methods of oil shale leaching would involve mining the shale before the leaching begins, although one researcher has suggested in-situ leaching.[1] Researchers have obtained a patent on a process that utilizes Thiobacillus, Ferrobacillus sulfooxidans, and rumen bacteria and a sulfuric acid solution to remove minerals from oil shale, leaving kerogen and bitumen.[1]

Waste Streams and Control Technologies--

Potential waste streams of microbiologically enhanced oil recovery processes are listed in Table 12. Very little data on this subject exist in the literature; most research and field efforts have focused on getting the processes to work rather than identifying possible environmental problems.

In general, waste streams for the microorganism and nutrient injection processes are similar and include: microorganisms, offgases, unused nutrients, substrate, and organic acids. Crude oil that is pumped to the surface will contain microorganisms and will have to be treated during the refining process. Other microorganisms may leach through permeable rock in the oil reservoir and migrate into soil and water. A study at the University of California showed that oil recovery enhancing bacteria can be transported in oil saturated porous media.[29] This presents a significant concern, since at least two types of bacteria (Pseudomonas and Acinetobacter) used for oil recovery already have been identified as microorganisms capable of biotransforming organic substances into compounds potentially harmful to humans.[25] Additional research on the behavior of these microorganisms in representative soils and waters is needed.

Offgases produced by tertiary oil recovery will be released into the atmosphere and will either be recovered as methane or treated by flaring as they currently are in oil drilling operations.

5.2.2 Direct Synthesis of Hydrocarbons by Microorganisms and Higher Life Forms

Microorganisms and plant forms such as rubber plants can synthesize liquid and gaseous hydrocarbons. Methane-producing bacteria found in soils already are used in the treatment of municipal sewage. They also produce ethylene, ethane, propane, and propylene. A wide variety of bacteria may utilize wastes in a fermentation-type process that probably would take place in a pond or other relatively contained structure. Some use glucose as their source of carbon; others use waste products.

TABLE 12. WASTE STREAMS/CONTROLS FOR MICROBIOLOGICALLY ENCHANCED OIL RECOVERY PROCESSES*

Process	Waste Stream	Potential Contaminants	Current Treatment/ Disposal Methods
Injection of Nutrients	Leachate from reservoir	Bio- or chemical transformation products in soil, organic acids, unused nutrients	No data
	Offgases**	methane carbon dioxide nitrogen H_2S	recovery flaring
Injection of Micro-organisms	Leachate from reservoir	Bio- or chemical transformation products in soil, organic acids, and unused nutrients and substrate	No data
	Offgases**	methane carbon dioxide nitrogen hydrogen sulfide	recovery flaring

*Based on bench and field test applications only.

**Offgases do not include gases typically emitted from oil production (e.g., light hydrocarbons).

Many species of algae produce hydrocarbon oils through photosynthesis. They extract carbon dioxide from air and use the energy of photosynthesis to reduce the CO_2 and polymerize it into long chain lipids.[25] Rubber trees similarly use photosynthesis to produce hydrocarbon oils.[1] Research on the use of giant kelp and other marine plants as an energy source has been taking place since the 1960s (e.g., brown algae and sargassum weed).[30]

None of the processes described above has been used on any significant commercial level. For the most part, these processes are envisioned as taking place in a controlled pond or tank environment to make harvesting easier.

Waste Streams and Control Technologies--

It is difficult to speculate on the waste streams of direct hydrocarbon synthesis processes since the details of these processes have yet to be fully resolved. Very little data are available on the processes themselves, not to mention the associated waste streams.

Similarly, no information exists regarding control procedures, other than through design of ponds and tanks. Gene manipulation has been mentioned only as a means of increasing the efficiency of a microorganism in synthesizing hydrocarbon products.

5.2.3 Landfill Gas Processes

Recovery of methane gas from landfills is another source of energy generated by biological processes. Figure 12 illustrates landfill gas processes. After refuse is deposited in a landfill the naturally occurring aerobic bacteria consume the oxygen present. Most oxygen is purged within a few weeks after the refuse has been placed in the landfill. Then, the oxygen-deficient environment results in growth of anaerobic bacteria which consume organic molecules. The large organic molecules are broken into smaller organics first, and eventually are decomposed to CO_2 and CH_4. Results of field tests indicate that gas production rates can be expected to range from 0.043-0.120 ft^3/lb of refuse per year with methane concentrations of

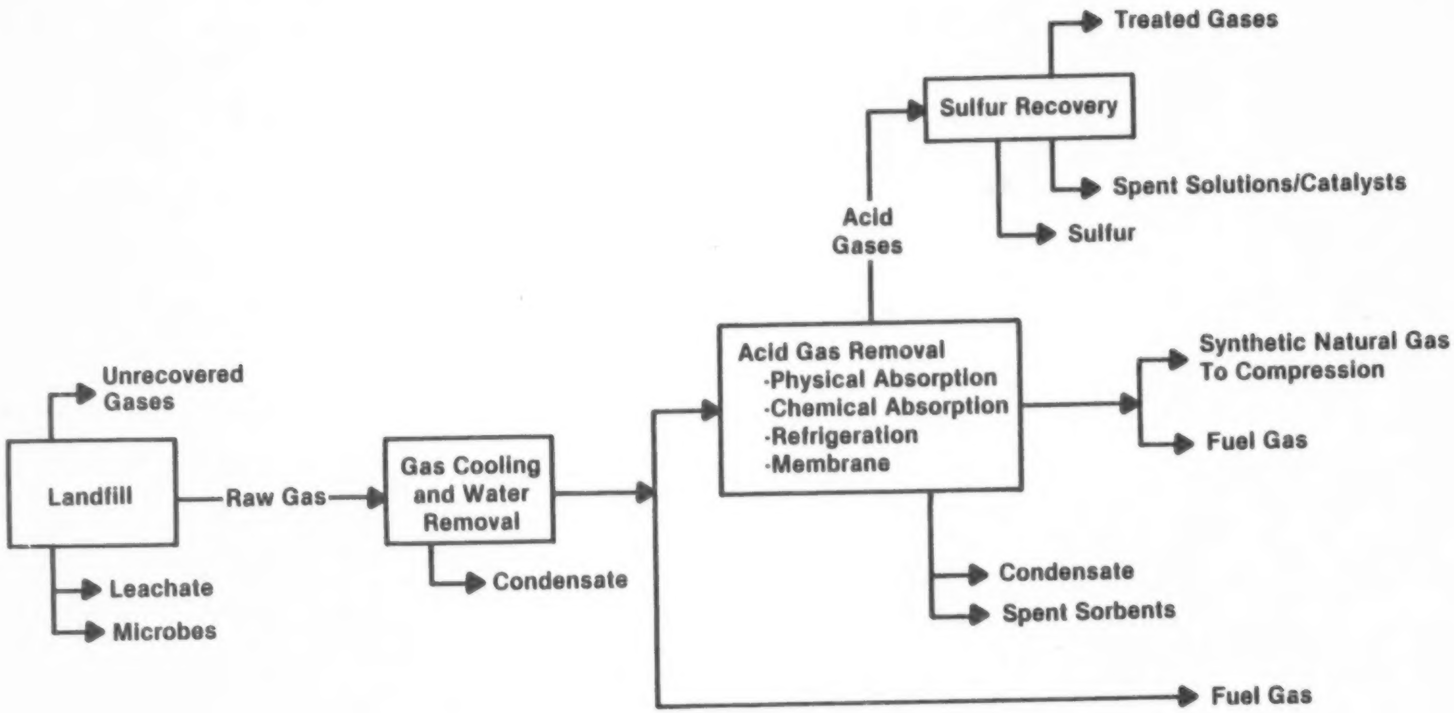

Unrecovered Gases
Landfill
Raw Gas
Leachate
Microbes
Gas Cooling and Water Removal
Condensate
Acid Gas Removal
·Physical Absorption
·Chemical Absorption
·Refrigeration
·Membrane
Acid Gases
Sulfur Recovery
Treated Gases
Spent Solutions/Catalysts
Sulfur
Synthetic Natural Gas To Compression
Fuel Gas
Condensate
Spent Sorbents
Fuel Gas

Figure 12. Landfill Gas Processes.

42-55 percent. Trace amounts of hydrogen and H_2S gases may also be produced if sulfur-bearing material is present. Ammonia and carbonate produced are dissolved in the water present in the landfill.

The major factors influencing gas formation are listed below:

- refuse composition (i.e., fraction biodegradable)
- moisture content
- bacterial population
- quantity and quality of nutrients for the bacteria
- temperature
- pH.

The refuse composition is typically not a variable in recovering landfill gas, as the landfill generally already exists. However, gas production from landfills where nondecomposable material has been separated should be higher than from other landfills.

Moisture content of the landfill has been found to be the most important enhancement parameter. It provides the aqueous environment in which the bacteria live and also serves as a medium for transporting the bacteria and their nutrients throughout the landfill. Moisture content is a variable which can be controlled in full scale systems. Methods include the following:

- inducing infiltration of rainwater
- injecting liquids (leachate recirculation or injection using wells)
- applying water during refuse placement.

The principal nutrients required for methane production are carbon, hydrogen, oxygen, nitrogen, and phosphorous. Also bacterial cells require specific concentrations of sodium, potassium, calcium, and magnesium. The most likely form of nutrient addition and bacterial population seeding is sewage sludge. Other sources include animal and agricultural wastes.

Temperature and pH also impact the gas formation rate. Methane-forming (methanogenic) bacteria require a narrow pH range of 6.7-7.2 as an optimum level. In full-scale systems pH control can be achieved by adding a buffer such as calcium carbonate (limestone) to neutralize the organic acids formed.

Bacterial population growth is sensitive to temperature. In landfills, the temperature typically varies from 35-60°C. The top layer may fluctuate with air temperature but lower levels (below 6 feet) typically do not fluctuate significantly.

Temperature management is not considered practical for full scale landfill gas recovery systems. In general, practices such as using an extra thick top soil layer for insulation are effective in winter but the effect is counterproductive in the summer. An insulating layer in the summer reduces the rate at which heat is lost, and methane production is lowered at high temperatures.

The most practical enhancement technique for improving gas production is moisture control. Water can be added to a landfill by trucks such as those used to spray water for dust control. Also, liquid wastes such as sewage sludge can be mixed in with the refuse before placement adding both nutrients and microbes in addition to moisture. The leachate generated from moisture addition can be recycled in landfills which have leachate collection facilities. Other enhancement techniques which improve methane production but may not be as practical as moisture addition include pH control through and particle size and refuse density control through operating procedures during refuse placement (e.g. shredding).

The current technology for recovering the gas generated in landfills is to use vertical extraction wells. A vacuum applied at the wellhead induces the gas within the landfill to flow up through the well.

Alternative recovery schemes primarily involve managing the landfill during placement of refuse to create permeable corridors for the gas to be collected. These permeable corridors may be gravel, or the selective use of broken pipe, tires, etc. at strategic locations can also provide relatively continuous voids. Still another possibility is to use a horizontal piping system laid underground far enough to prevent significant air intrusion. This could be supplemented by using an impermeable liner such as plastic film on the surface of the fill.

One common end use for landfill gas is to sell it to a pipeline company or some nearby industrial consumer. However, the landfill gas may require cleanup before it can be used. Carbon dioxide and H_2S removal may be required in addition to moisture removal to produce pipeline quality gas. The processing steps which may be included in gas cleanup are illustrated in Figure 12.

The alternative to cleaning the landfill gas and selling the resulting pipeline quality gas is to use the gas on-site to generate electricity. The gas would not require extensive cleanup since it will be combusted on-site. Either an internal-combusion engine or gas turbine with a generator and other electrical auxiliaries can be used directly. The electricity can then be sold to local users or a power company.

The wastestreams and potential contaminants associated with landfill gas processes are presented in Table 13. Wastes from the site itself include uncollected gases and leachate. The gases may contain reduced sulfur and nitrogen species, volatile organics and possibly halogenated organics. These emissions may be controlled through the use of efficient gas collection systems.

Leachate produced in the landfill could contain a variety of organics, dissolved gases, and metals depending on the characteristics of the wastes buried in the site. The leachate can also provide a method of transport for microorganisms.

TABLE 13. WASTE STREAMS CONTROLS FOR LANDFILL GAS PROCESSES

Process Unit	Waste Stream	Potential Contaminants	Current Methods of Control
Landfill	Uncollected Gases	Reduced sulfur/nitrogen species, volatile organics and possibly halogenated organics	Control by gas collection system
	Leachate	Organics, trace metals, dissolved gases, microbes, possibly halogenated organics, microorganisms	No data
Gas Cooling and Water Removal	Condensate	Same as leachate	No data
Acid Gas Removal	Acid gases	Reduced sulfur/nitrogen species, volatile organics, possibly halogenated organics	Sulfur control combustion
	Condensate	Dissolved gases, spent solvent, organics, possibly halogenated organics	No data
	Spent sorbents and catalysts	Trace metals, organics	Recycle to vendor regenerate
Sulfur Recovery	Treated gases	Reduced sulfur/nitrogen species, volatile organics, combustion gases	Not applicable
	Spent sorbents	Trace metals, organics	Recycle to catalyst vendor Disposal as a hazardous waste
	Sulfur	Trace metals, organics	Landfill Salable by-product

Several wastestreams are associated with the subsequent gas purification processes. The condensate formed when the raw gas is initially cooled would be expected to contain similar contaminants to the leachate.

Acid gas removal processes can result in an acid gas wastestream containing reduced sulfur/nitrogen species and volatile organics. The condensate formed in these processes may contain dissolved gases, spent solvents, organics and potentially halogenated organics. Absorption acid gas removal processes will also generate spent sorbents contaminated with trace metals and organics.

Sulfur may be recovered from the acid gases removed from the fuel gas. These sulfur recovery processes generate spent sorbents and catalysts contaminated with trace metals and organics.

5.3 POLLUTION CONTROL

The use of microorganisms for pollution control is not a new concept. Microorganisms are widely used to treat municipal sewage and other industrial wastes. Because this technology is used in a relatively contained environment and is already addressed by current pollution control guidelines, this use is not discussed in this report.

More recently, it has been found that microorganisms can play an important role in removal of hazardous organic compounds including petroleum hydrocarbons, toxic heavy metals, and high sulfur and high nitrogen effluents. At this time, most of these processes are in the development stage or early stages of commercial use.

5.3.1 Hazardous Organic Substance Removal

The area which currently appears to have the most potential for commercial use is biological treatment of organic hazardous substances. This treatment consists of biotransformation, biodegradation, and decomposition of organic compounds by a large number of microorganisms. It can take place in-situ (on land or possibly at sea) or in tanks or reactors into which ground water, for example, could be pumped.

Researchers at the Advanced Environmental Control Technology Research Center at the University of Illinois performed an in-depth evaluation of the ability of microorganisms to remove man-made organic compounds, mainly priority pollutants, from soils and waters.[31] They identified a wide variety of microorganisms capable of biodegrading, biotransforming, accumulating, and decomposing almost every class of man-made compound. These microorganisms include actinomycetes, fungi, bacteria (including anaerobic and oligotrophic), and photosynthetic microorganisms (such as algae).

Actinomycetes attack a large group of organic compounds including phenols, pyridines, glycerides, steroids, chlorinated and nonchlorinated aromatic compounds, and lignocellulose. Kobayashi and Rittman concluded that actinomycetes appear to be especially useful in treating contaminated soil where composting is used.[31]

Two orders of fungi show the most promise for organics removal: Mucorales and Moniliales. They generally are believed to be able to biodegrade PCBs better than bacteria. One type of Moniliales--*Fusarioum oxysporum*--can completly degrade DDT.

Bacteria that can degrade or remove organics can be isolated from areas of hydrocarbon contamination. These are heterotrophic genera including *Pseudomonas*, *Achromobacter*, *Arthrobacter*, *Micrococcus*, *Vibrio*, *Acinetobacter*, *Brevibacterium*, *Corynebacterium*, and *Flavobacterium*. Photosynthetic microorganisms such as algae and photosynthetic bacteria cause transformations and are able to bioaccumulate hydrophobic compounds.

For all of these microorganisms the feasibility of biodegradation/biotransformation depends on interactions among factors such as dissolved oxygen, temperature, pH, salinity, and the presence of competing organisms. In addition, Kobayashi and Rittman stress the importance of the selection, development, and retention of the microbial population in preparation of microbial cultures for use in biodegradation or biotransformation processes.[31]

In addition to laboratory research studies such as the one just described, a number of companies already are manufacturing and using microbial cultures to remove chemical wastes, lipids, detergents, cellulose, and crude oil from soil and water. Polybac Corporation of Allentown, Pennsylvania, manufactures a product that degrades a large number of hazardous compounds in-situ, including pentachlorophenol.[32] The company currently is developing microbial products to remove PCBs and heavy metals as well. Other companies are developing mutated bacterial cultures to degrade dioxins.[33] These and similar products may be applied to contaminated soil to reduce residual pollutants.

Waste Streams and Controls--

Very little information is available on potential waste streams caused by use of these pollution control technologies. In some instances pollutant removal may take place in a controlled tank; more often it will occur at the site of an accidental spill or at a hazardous waste disposal site. Further study is needed to gain more insight into the metabolic activity and migration potential of microorganisms used for pollution control. Information about the biotransformation capabilities of some of these microbes will be crucial to controlling process waste streams.

5.3.2 Desulfurization/Denitrification

Oxidized forms of nitrogen (NO_x) and sulfur (SO_2) formed during the combustion of fossil fuels present serious environmental problems. Chemical, physical, and bacterial coal cleaning processes have been investigated as a means of alleviating this problem by removing sulfur or nitrogen before the fuel is burned.

Bacterial coal desulfurization is actually the oxidation of coal pyrite by autolithotrophic bacteria of the *Thiobacillus*, *Ferrobacillus*, or *Sulfobolus acidocalderious* strains. Sulfur in coal exists as sulfates, sulfides, and organic sulfur compounds. The sulfur is leached from the coal pyrite by the bacteria as metals are leached from ore. The bacteria generally are suspended in a sulfuric acid solution prior to application to a coal slurry.[1] Coal desulfurization by microorganisms has only been tested in the laboratory, but appears promising for desulfurization of concentrated coal-water or coal-alcohol slurries.[34]

Removal of sulfur from oils makes use of strains of *Thiobacillus* isolated from crude oil-soaked soils, seawater, industrial waste, etc., which convert organic sulfur into water soluble compounds. Like desulfurization of coal, this process is still in the developmental phase.

Microbial denitrification of fossil fuels to remove excess nitrogen and ammonia has not yet been widely investigated. It probably would make use of the Pseudomonas strain of bacteria.

Waste Streams and Controls--

Since desulfurization and denitrification processes are still experimental, almost nothing has been written about their waste streams and possible controls. However, desulfurization processes produce sulfuric acid (similar to metal leaching processes), a potential contaminant if not carefully controlled. At this point it appears that many of these desulfurization and denitrification processes will take place in a relatively contained environment (e.g., a tank or pipeline) rather than in an open dump.

SECTION 6

POTENTIAL ENVIRONMENTAL IMPACTS AND CONTROL TECHNOLOGIES ASSOCIATED WITH BIOTECHNOLOGY WASTE STREAMS

Potential environmental impacts associated with biotechnology processes center around the discharge of high strength organic waste streams, particulate and volatile organic emissions, and the release/disposal of viable microorganisms. The following sections present the potential adverse environmental impacts associated with both chemical and biological constituents in biotechnology process waste streams as well as a discussion on the effectiveness and appropriateness of current control technologies. Areas for which adequate data are not available on the presence and potential impact of waste stream constituents, the control technology currently used and the effectiveness of control technologies are identified.

6.1 IMPACTS OF CHEMICAL CONTAMINANTS

6.1.1 Liquid Waste Streams

Sources of chemical constituents in biotechnology liquid waste streams include unused organic substrates, residual product, intermediate products and by-products, nutrients, and a variety of chemical compounds and trace elements which may have been introduced with the substrate or in product recovery and purification processes. Potential substrates and products include a wide range of organic chemicals; process substrates may also include agricultural and forestry residues, and grains. In general, process effluents are high in BOD, COD, and TSS and may contain refractory organics, organic acids, alcohols, hydrocarbons, metals, pesticides from cellulosic substrates and biocides from equipment cleaning. The specific chemical constituents associated with the substrate and the product will be dependent on the specific process and the conditions of the process. Because of the highly proprietary nature of most fermentation processes and the lack of commercial-scale applications of uncontained processes, little or no data are available as to the specific characteristics of these waste streams. This lack of data applies to biotechnology waste streams as a whole; however, some specific areas with noticeable data gaps are as follows:

- Characteristics of waste streams associated with enzyme and/or acid hydrolysis of agricultural and forestry feedstocks. These streams may contain pesticides and in the case of forestry feedstocks contain a lignin-rich waste with high concentrations of phenols and other aromatic hydrocarbons.
- Characteristics of liquid waste streams associated with substrate and fermentation vessel sterilization. No data were found on the frequency and volume of such discharges. Additionally, these streams may contain chemical biocides and detergents used in sterilization processes.
- Characteristics of liquid waste streams associated with enzyme or cell immobilization processes.
- Characteristics of liquid waste streams associated with product recovery and purification processes. These waste streams are generated from a wide variety of processes and typically represent the largest source of waste streams.
- Characteristics of liquid waste streams resulting from noncontained process applications such as microbial mining or enhanced oil recovery. Most of these applications are in the developmental stage and very little is known about the potential waste stream constituents and their fate in the environment.

If the potential environmental impact of these waste streams is to be determined, much more detailed information on the waste stream characteristics will be needed. Discharge of high BOD/COD wastewaters can cause numerous adverse environmental impacts including decreases in receiving stream dissolved oxygen concentration, fish kills, and advanced eutrophication. Additionally, specific organic and inorganic contaminants may be toxic to humans and/or wildlife, or reduce the potential for reuse of process wastewaters. For example, refractory organics such as humic and fulvic acids can:

- react almost irreversibly with strong base ion exchange resins used to remove ions from water for boiler feed water;
- degrade in boilers to form corrosive compounds;
- form silicate complexes that can deposit on turbine parts and boiler tubes;
- form stable complexes with heavy metals, thereby increasing the solubility of those metals;
- form soluble complexes with pesticides, insecticides, and hydrocarbons; and
- be potential precursors to the formation of halogenated hydrocarbons.

6.1.2 Solid Waste Streams

Solid waste streams from biotechnology processes include spent biomass, sludges from wet air scrubbers, and waste sludges from biological wastewater treatment processes. Additionally, product recovery and purification processes may also generate spent solvents and solid or sludge residuals. As was the case for process effluents, little or no specific data were found as to the identification and characterization of solid waste streams.

Chemical constituents of solid waste streams would be expected to include those contaminants discussed in process effluents (e.g., residual organics, metals, pesticides). The potential concentration of these contaminants, however, is unknown. While it is reported that biological sludges from certain fermentation processes (e.g., sludges from ethanol production) are nontoxic,[35] this assumption cannot be made for all fermentation processes as production of some products could introduce refractory organics and metals which can accumulate in biological sludges. Additionally, other wastewater treatment technologies can produce filter cakes or sludges with high concentrations of contaminants such as heavy metals. Uncontrolled disposal of the solid wastes associated with biotechnology processes could result in migration of these contaminants in the environment.

6.1.3 Gaseous Waste Streams

A major problem in the fermentation industry is the production of aerosols from the fermenter and product recovery and purification processes such as centrifugation. Aerosols provide an excellent method of transport for chemical and biological constituents which may be harmful to human health and the environment.

Other gaseous emissions from biotechnology processes consist primarily of particulates from preparation of agricultural feedstocks and drying of spent biomass by-product, fermentation offgases, and emissions from product recovery and purification processes such as distillation, steam stripping and evaporation. Atmospheric emissions associated with auxiliary processes, such as steam generation, are the primary sources of criteria pollutants; however, these sources are not discussed in this report as they are not unique to biotechnology processes.

Few data were found on the characteristics of biotechnology air emissions. Fermentation offgases will have high concentrations of CO_2 or methane, depending on whether the process is aerobic or anaerobic, respectively. These offgases may also contain traces of volatile organic carbon (VOC). Similarly, emissions from product recovery processes may also contain VOC. Particulate and VOC emissions can pose a threat to the health of plant workers, and odor problems are often associated with fermentation offgases.

6.2 IMPACTS OF MICROBIAL CONTAMINANTS

The major concern in biotechnology processes is the potential for harmful effects from the release of viable microorganisms both in the workplace and in the general environment. In contained process applications, viable microbes may be released in all of the fermentation or product recovery processes' waste streams or in the product itself. Uncontained processes deliberately release the microbe directly to the environment. The concerns are especially heightened when genetically altered microbes are used.

The hazards associated with release of viable microbes include adverse effects of the microbe or its products on the natural balance, allergic reactions in plant workers, the potential for some organisms to cause disease in higher life forms, and the potential for the organism to change during the process or after release thus invalidating previous testing which demonstrated the process's safety.

In the case of organisms which are not pathogenic, the concerns relate mainly to environmental impacts. The discharge of viable organisms may cause competition which could alter the natural balance with no predictable result.[27] Traits of the process microbe may be transferred to naturally occurring species via bacterial conjugation, transduction, or transposition. The microbe may also excrete undesirable secondary metabolites, such as antibiotics, thus resulting in the development of resistant strains of organisms. Additionally, the use of plant pathogens on the commercial scale is increasing. Release of such microbes could increase damage to crops; and, because such microbes are ideally suited for transport by aerosols, they could spread the damage over extensive and distant areas.[27]

Microorganisms or the enzymes they produce can cause allergic reactions in humans. Workers in plants producing citric acid have experienced a Farmers Lung type of allergy, and workers contacting certain enzymes in detergent powder manufacture have also experienced allergic reactions.[27] The presence of low levels of enzymes in products such as soft drinks and fructose is also of concern as this might have long-term sensitizing effects.[27]

The use of pathogenic organisms presents the potential for more serious hazards, particularly in the workplace. Because of the ability of microorganisms to reproduce and multiply, even the smallest release could be potentially hazardous.[4] The microbe itself may produce disease symptoms, or it may produce toxic substances with the degree of hazard dependent on the method of exposure, dose, and the condition of the recipient. Additionally, organisms not normally considered pathogenic may produce harmful effects if the dose is sufficiently large, and genetically manipulated microorganisms could conceivably present a serious threat. Numerous pathogenic organisms occur naturally in the environment, and their control is somewhat understood. The new concern presented by biotechnology is the large scale on which some organisms are grown and the large number of people who may encounter the organism either in the workplace, through the use of the product, or from release to the environment.

Perhaps one of the major uncertainties associated with the use of microorganisms, especially geneti[illegible]ly engineered microbes, is the potential for their characteristics to change during process application or after release to the environment. If a contaminated or changed organism does occur and is able to survive, then regardless of any original testing, no guarantee concerning the process safety can be given. The virulence, or capacity of an organism to cause disease, may be enhanced by phage (virus) transduction, and variations may be induced by a variety of changes in growth conditions such as growth method (batch vs. continuous), growth rate, nutrient and trace element supply, temperature, and pH.[27] Further, natural mutations will occur and may be enhanced if the culture is contaminated with foreign organisms. This is of more concern in continuous fermentations than in batch processes as the culture may be sustained for many thousands of generations.[27]

It is conjectured that the highly specialized organisms used in industrial processes would have little chance of survival outside of the controlled environment of the fermentation vessel. Additionally, through recombinant DNA techniques, enfeebling mechanisms could be programmed into the cell to ensure its demise in the open environment. However, the ability to survive is a well known trait of microbes and is a desirable trait from the process application perspective, especially in those processes involving direct release.

The full extent of potential hazards associated with release of any viable microorganism and in particular genetically engineered microorganisms is generally unknown. In order to assess the degree of controls required to treat or contain the microbe, a realistic hazard assessment for microbial release is needed. This procedure for evaluating microbial impacts to human health and the environment would assess the conditions of the process in which the microbe is to be used and the characteristics of the microbe such as:

- infectivity/invasiveness,
- pathogenicity/virulence,
- acute and chronic toxicity,
- irritancy,
- hypersensitivity,
- immune responses,
- reproductive capacity,
- persistence, and
- adaptability.

The results of such an assessment would define appropriate levels of precaution both for the workplace and for the general environment.

6.3 ENVIRONMENTAL CONTROL TECHNOLOGIES

Most environmental control technologies currently employed in biotechnology processes are aimed at treatment of chemical contaminants in waste streams as opposed to control of biological contaminants. The following sections discuss the technologies currently used for control of chemical and microbial contaminants.

6.3.1 Control/Treatment of Chemical Contaminants

Liquid Waste Stream Treatment--

Biotechnology liquid waste streams are currently treated using conventional biological treatment processes with pretreatment of isolated waste streams for specific pollutant removal as required. Such pretreatment processes may include cyanide destruction via chlorination, ozonation, or acid hydrolysis; metals removal by chemical reduction or precipitation; and solvent recovery or treatment by steam stripping or carbon adsorption.[2] Biological treatment is primarily aimed at reduction of the high BOD/COD concentrations associated with process wastewaters. Many existing ethanol production facilities employ extended aeration biological treatment because of its ability to treat effluents with high organic loadings and its resistence to organic/inorganic shock.[35]

Very little data were found as to the effectiveness of biological treatment on process waste streams. In general, biological treatment should be adequate for control of BOD/COD. Well designed and operated solids removal units should also control TSS. The effectiveness of this treatment on specific organic or metal pollutants, however, will be highly variable depending on the specific design of the process. Some potential problems associated with biological treatment of these wastes include:

- Refractory organics and/or metals will probably pass through the system untreated or accumulate in the sludge.
- Some chemical constituents, such as chemical biocides used in the sterilization process, may inhibit the system thus reducing treatment effectiveness. These constituents will exit the treatment process in the effluent or sludges.
- The periodic discharge of large volumes of contaminated fermentation batches could result in shock loadings to the system unless adequate equalization is achieved.
- The wastewaters may contain high concentrations of nutrients which may require treatment prior to discharge.
- The presence of the process microbe could inhibit the system through competition with other microorganisms in the mixed liquor or by producing substances inhibitory to the mixed liquor bacteria.

Many process waste streams (e.g., spent fermenter broth) may potentially be recycled to the process; however, the extent of the application of recycle in the industry is not known. Spent fermenter broth will contain nutrients valuable to the process; however, contaminants destructive to the process may be introduced to the broth during product recovery and purification processes. Additionally, the waste stream would require sterilization prior to reuse.

Gaseous Waste Stream Control--

Control of gaseous emissions consists primarily of:

- particulate controls on emissions from substrate pretreatment and spent biomass drying,
- treatment of fermentation offgases and aerosols, and
- treatment of emissions from product recovery and purification processes.

Controls used for particulate removal include cyclones, baghouses, and wet scrubbers. Cyclones and baghouses are primarily used for treatment of particulates from substrate preparation. Wet scrubbers are reported to be better suited to exhaust gases from spent biomass drying since these particulates easily cake on fabric filters decreasing their effectiveness. Liquid waste from these scrubbers are usually routed to wastewater treatment.[35]

Currently, fermentation offgases from aerobic processes are often vented directly to the atmosphere or alternatively may undergo cryogenic CO_2 recovery. In cases where this waste stream poses a severe odor problem the vented offgases may be incinerated, which has the added advantage of controlling VOC emissions. Prior to CO_2 recovery, impurities are removed by passing the gas through a wet scrubber with the liquid waste routed to wastewater treatment. Such processing may also remove some VOC contaminants. The extent to which VOC controls are needed will be dependent on the VOC species contained in these emissions. Fermentation offgases from anaerobic processes consist primarily of methane and, therefore, may be recovered as a by-product or flared.

While it is stated that aerosol emissions are a primary concern in fermentation processes, very few data were found addressing the control technologies for this emission. Potential control technologies may include use of anti-foaming agents or filtration of offgases; however, current use and the effectiveness of these controls are unknown.

Dilute VOC emissions from product recovery processes may be incinerated, or flared, or utilized as air feed to process boilers.[35] As previously stated, very little data are available as to actual current practice in the industry.

Solid Waste Stream Control--

The largest solid waste stream from fermentation processes is the spent biomass. Control of this waste is discussed in the section on control of microbial constituents. Sludges from biological treatment are primarily landfilled, dried and landfarmed, or may undergo anaerobic digestion with methane recovery and use of the digested sludge as animal feed or fertilizer. Most disposal practices assume that these sludges are non-toxic; however, as previously stated, they may contain accumulated organic and metal contaminants from process effluents.

Other sludges generated from pollution control technologies such as metals precipitation may contain toxic levels of pollutants requiring disposal under the Resource Conservation and Recovery Act (RCRA). Waste solvents, if disposed of, must adhere to RCRA requirements; however, this waste may be recovered for subsequent reuse.

6.3.2 Control/Treatment of Microbial Contaminants

Control of the microbe typically involves containment, appropriate operator protection, and may include detoxification of gaseous emissions, liquid effluents, and solid wastes. In regards to specific microbes, zero discharge may be required in cases of known extreme toxicity or hazardousness. Most currently used controls are aimed at treatment of chemical contaminants as opposed to treatment of the microbe. The following subsections discuss the effectiveness, if any, of these conventional controls on the microbe as well as potential effects of the microbe on the performance of these processes. Subsequently, control technologies specifically aimed at control of the microbe are discussed.

Effectiveness of Conventional Waste Treatment Technologies on Control of the Microbe--

Biological wastewater treatment technologies essentially have no effect on the microbe. Process microorganisms would be part of the mixed liquor and discharged to the environment with the waste sludge and in the control process effluent. No data were found on the potential impact of the process microbe on the treatment process. However, the presence of the process microbe could inhibit the system through competition with other microorganisms in the mixed liquor or by continuing to produce the organic product or other metabolites which may be inhibitory to the mixed liquor bacteria.

Similarly, the presence of the microbe may change the wastewater matrix in such a way as to interfere with other physical/chemical wastewater treatment technologies. For example, lipid cell matter released during cell disruption for product recovery could prevent agglomeration of precipitated solids or decrease the settleability of precipitates formed in metal precipitation processes.

Controls for particulate emissions also would do little to hinder the release of the microbe as such controls are typically not designed to remove particles as small as microorganisms. A similar argument would hold for filtration of fermenter offgases. These filters are most probably not designed to remove microorganisms but rather to prevent gross emissions of aerosols.

Incineration of vented gases could result in destruction of the microbe if the system were designed to achieve sterilization. However, the extent to which incineration of offgases is practiced is unknown. Flares designed for the destruction of residual VOC may result in some destruction of the microbe;

however, aerosols produced in flaring of low VOC emissions could provide a method of transport of the microbe to the environment. Wet scrubbing of gaseous emissions would have little effect on the microbe other than to, perhaps, transfer the contaminant from the gas stream to the scrubber sludge and blowdown.

Solid waste disposal practices also typically do not address the presence of the microbe as a contaminant. Landfilling and landfarming of sludges containing the process microbe represent direct release of the microbe to the environment.

Methods to Specifically Control/Treat Microbial Contaminants--

As the above section indicates, conventional waste treatment technologies do little to control release of the microbe. Additionally, few data were found in the literature addressing this issue.

Spent biomass from fermentation processes represent the largest volume and highest concentration of microbial waste. In ethanol production, current industry practice is to dry and market spent biomass as animal feed. Alternatively, the spent biomass may undergo anaerobic digestion for methane recovery with the residuals used as fertilizer or animal feed. It is highly unlikely that all, or even most, spent biomass wastes will be appropriate for use as animal feed due to potential hazards associated with the microorganism itself or due to residual chemical contaminants. In such cases, technologies for treatment and/or disposal of this waste may be required.

Currently, when large quantities of culture are discharged, the viability of the wastes may be reduced by chemical treatment (e.g., chlorination), heat sterilization, or incineration. Chlorination, however, can result in the formation of chlorinated hydrocarbons from residual organic contaminants in the spent biomass and incineration is relatively expensive because of the high moisture content of biomass filter cakes and sludges.[4] No data were found as to the effectiveness of these controls in large-scale processes.

Spent biomass is not the only waste stream in which the microbe will appear as a contaminant. Because of the nature of submerged fermentation processes and the pervasiveness of microorganisms, virtually all waste streams can be expected to contain the process microbe to some degree. Current industry practice requires treatment of these waste streams for control of the microbe when it is known to be of an infectious nature. The methods most commonly used in the pharmaceutical industry are autoclaving and incineration.[2] It should be noted, however, that many waste streams from the production of medicinals are relatively low volume wastes. The use of these processes on high volume waste streams may not be effective or economical.

Containment technologies are also employed to prevent release of the microbe. These technologies may range from basic enclosure to the use of filters capable of retaining the microbe and complete isolation. In some cases these technologies may be employed to prevent release within the plant but may not prevent ultimate discharge to the environment. For example, fermentation offgases may be vented out of the immediate plant area because workers have experienced allergic reactions but the vented offgases are then discharged to the environment.

An alternative form of containment is the use of immobilized cells or enzymes in the fermentation process. The use of these processes could greatly reduce the concentration of microbes and enzymes in the waste streams from the fermenter. However, organisms and enzymes can "leak" from the support or encapsulation matrix resulting in some degree of contamination in the process discharge streams.

Another proposed control technology to reduce the impact of microbial release is the use of organisms genetically engineered with an enfeebling mechanism that would prevent their survival in the open environment. For instance, the microbe could be engineered in such a manner that it required a very specific combination of nutrients to survive. Theoretically, once released to the environment and denied this source of nutrients the organism would die. Microorganisms, however, are highly adaptive and once denied a given nutrient source the organism may simply adapt to utilize available nutrients. Additionally, introduction of such enfeebling mechanisms may not be compatible with process considerations as it would require more stringent process control to ensure the survival of the microbe in the fermenter.

Nutrient denial as a treatment prior to discharge of the microbially contaminated waste stream may also be used as a control technology; however, no discussion of this technology was found in the literature.

While control technologies to contain or treat the microbe are available, the extent of their effectiveness and their applicability to large-scale processes is unknown. The extent of use and the need for stringent controls will be dependent on the development of reliable methods to monitor microbial releases and to assess the risks associated with those releases. Quantification of these risks would aid in defining the appropriate level of control as well as provide a framework from which to evaluate current controls.

SECTION 7

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SECTION 8

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SECTION 9

GLOSSARY

Actinomycetes - a member of the bacterial family Actinomycetaceae. Includes chemoorganotrophs that ferment carbohydrates.

Affinity separation agents - separation agents which utilize the ability of the biological molecule to bind other compounds or molecules specifically and reversibly such as in affinity chromatography.

Antigen - any chemical compound, usually of large molecular weight such as a protein or carbohydrate, that stimulates the production of antibodies.

Codon - a three nucleotide sequence on a DNA chain that codes for a particular amino acid.

Collagen - a fibrous protein found primarily in the connective tissue of multicellular animals.

Frameshift - mutation which occurs when one or more nucleic acid base units are deleted.

Genotypic - relating to the genetic makeup of an organism, usually in regard to one or a few genes.

Hybridoma - clone of cells which produce monoclonal antibodies.

Kaolin - any of a group of clay minerals that have a two-layer crystal in which silicon-oxygen and aluminum-hydroxyl sheets alternate.

Ligase - an enzyme that catalyzes the formation of bonds which link together sements of single-stranded DNA.

Liposomes - particles of lipoid (fat) matter held emulsified in the tissues in the form of invisible fat.

Lymphocytes - antibody-producing cells formed in the lymphatic system of higher animals.

Lyophils - microbial cultures preserved by freeze-drying techniques.

Myeloma cells - a type of malignant tumor of the immune system which is used in the production of monoclonal antibodies.

Oligotrophic - having the ability to survive in the absence of nutrients.

Prokaryote - a group of organisms which include the bacteria and blue green algae that are characterized by the lack of a defined nucleus and the presence of a single chromosome.

Phage - a virus that infects bacterial cells.

Phenotypic - relating to the observable characters of an organism.

Plasmid - a small circular segment of double-stranded self-replicating DNA which is not part of the bacterial chromosome but is readily transferred between different strains of bacteria.

Protoplast - a bacterial or plant cell which lacks a cell wall.

Purine - a nitrogenous base component of nucleic acid containing a pyrimidine base fused to an imidazob ring.

Pyrimidine - heterocyclic nitrogenous base which is the structural component of a nucleotide or nucleic acid such as cytosine and thymine which are found in the DNA molecules.

Restriction endonuclease - a class of bacterial enzymes that cut both strands of DNA at specific sequences.

Rumen bacteria - bacteria found in the first stomach of a ruminant animal such as a cow.

Slants - microbal cultures grown on solid media.

Vector - The vehicle or agent which carries the gene into the host.

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